



TITLE:

# Studies on the Boron-Polysaccharide Complex of Higher Plant Cell Walls( Dissertation\_全文 )

AUTHOR(S):

Kobayashi, Masaru

---

CITATION:

Kobayashi, Masaru. Studies on the Boron-Polysaccharide Complex of Higher Plant Cell Walls. 京都大学, 2000, 博士(農学)

ISSUE DATE:

2000-11-24

URL:

<https://doi.org/10.11501/3176472>

RIGHT:

# **Studies on the Boron-Polysaccharide Complex of Higher Plant Cell Walls**

**Masaru Kobayashi**

**2000**

# Contents

<b>Introduction</b>	<b>1</b>
<b>1 Localization of Boron in Cultured Tobacco Cells</b>	<b>7</b>
1.1 Introduction . . . . .	7
1.2 Materials and methods . . . . .	7
1.3 Results and discussion . . . . .	9
<b>2 Structure of the Boron-Polysaccharide Complex of Radish Roots and Cultured Tobacco cells</b>	<b>13</b>
2.1 Introduction . . . . .	13
2.2 Materials and methods . . . . .	13
2.3 Results and discussion . . . . .	16
<b>3 Reconstitution of the Borate-Rhamnogalacturonan II Complex <i>in Vitro</i></b>	<b>25</b>
3.1 Introduction . . . . .	25
3.2 Materials and methods . . . . .	25
3.3 Results . . . . .	26
3.4 Discussion . . . . .	32
<b>4 Physiological Function of the Borate-Rhamnogalacturonan II Complex</b>	<b>35</b>
4.1 Introduction . . . . .	35
4.2 Materials and methods . . . . .	35
4.3 Results . . . . .	36
4.4 Discussion . . . . .	40
<b>Conclusion</b>	<b>43</b>
<b>Acknowledgements</b>	<b>45</b>
<b>References</b>	<b>47</b>
<b>Publications</b>	<b>53</b>



# Abbreviations

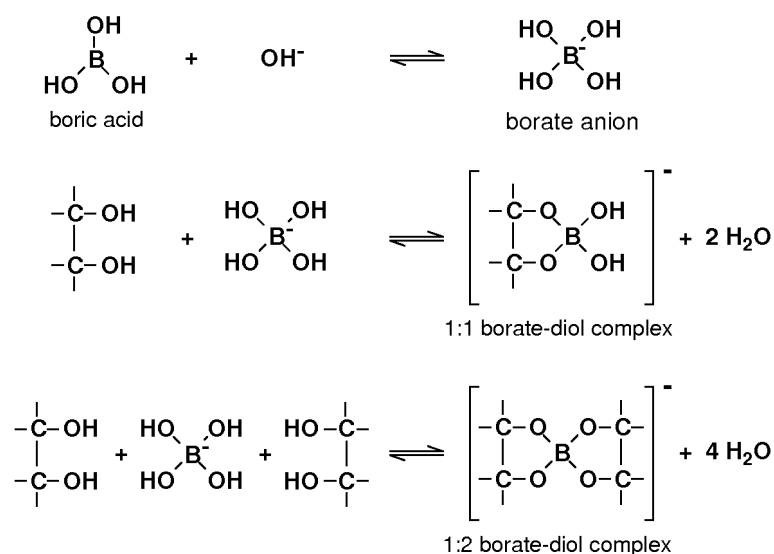
BPC	boron-polysaccharide complex
CDTA	<i>trans</i> -1,2-diaminocyclohexane- <i>N,N,N',N'</i> -tetraacetic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
GLC	gas-liquid chromatography
GLC-MS	gas-liquid chromatography-mass spectrometry
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
KDO	3-deoxy-D- <i>manno</i> -2-octulosonic acid
MES	2-morpholinoethanesulfonic acid
NMR	nuclear magnetic resonance
PGA	polygalacturonic acid
RG-I	rhamnogalacturonan I
RG-II	rhamnogalacturonan II
RID	refractive index detector
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane

# Introduction

Boron (B) is an essential microelement for vascular plants. Diatoms and some species of marine algae also require B, while most bacteria, fungi, green algae and animals apparently do not (Loomis and Durst 1992). The absolute requirement for B in plant nutrition was first demonstrated in broad bean (*Phaseolus vulgaris*) by Warington (1923). Although at that time she was not able to show a B requirement by graminaceous monocots, Sommer and Lipman (1926) demonstrated the requirement by barley (*Hordeum vulgare*) using repurified nutrient salts and redistilled water. These studies have been extended to many other plant species by a number of investigators, and B is now recognized as an essential micronutrient for higher plants in general. However the physiological function of B in plant is still the least understood of all the mineral nutrients.

Boron belongs to the metalloid or semiconductor family of elements, which also includes germanium. These elements are intermediate in properties between metals and non-metals, and also share many features in common in plants. Germanic acid partly substitute for boric acid in B-deficient plant (Skok 1957, Loomis and Durst 1992). Boric acid is very weak acid ( $pK_{a1}$  9.42), and occurs mainly as undissociated form in aqueous solution at  $pH < 7$ . At higher pH boric acid acts as a Lewis acid, which accepts hydroxyl ions from water and thus leaving an excess of protons (Fig. 1). Boric acid has a capacity to form ester compounds with diols and polyols, particularly with *cis*-diols (Fig. 1). The interconversion between boric acid, borate anion and borate esters occurs reversibly and spontaneously in aqueous solution (Henderson et al. 1973). As plant cell has many compounds with *cis*-diol such as polysaccharides, glycoproteins, nucleotides or *o*-diphenolic compounds, it is postulated that B fulfills its physiological function by forming complexes with those compounds.

Plant species vary significantly in their B contents, which generally reflects their requirement for B. The critical deficiency range is reported to be 20-70 mg per  $kg^{-1}$  dry



**Figure 1** Interconversion of boric acid, borate anion and their diol esters

weight in most dicotyledonous species, while graminaceous monocots require only 5-10 mg kg<sup>-1</sup> (Marschner 1995). Plants absorb B from soil solution in a form of boric acid. There is still a controversy as to whether boric acid could be taken up actively (Hu and Brown 1997). However, at least the major fraction should be taken up passively along with the transpiration stream, because the absorption rate is significantly affected by relative humidity, temperature and light intensity (Gupta 1993). Boron is immobile in plants, although the possibility of the retranslocation have been proposed for some species (Brown and Shelp 1997). The limited mobility may be ascribed to the association of B to the cell wall as borate-diol esters. Consequently B must be supplied throughout the growth period continuously, and the deficiency symptoms appear first in developing tissues such as root or shoot apex.

The most rapid response to B deficiency is inhibition or cessation of root elongation (Kouchi and Kumazawa 1975). In shoots terminal buds or youngest leaves cannot fully develop and may die in a severe case. Inhibition of internode elongation gives the plants a characteristic rosette-like appearance. Reproductive growth is also inhibited significantly by B deficiency, as it induces drop of buds, flowers or developing fruits. In addition, pollen

tube growth is particularly sensitive to B deficiency (Schmucker 1933).

What is known about B requirement so far arises mainly from studies of what happens when B is withheld or resupplied after deficiency. Parr and Loughman (1983) summarized the postulated functions of B including sugar transport, cell wall synthesis, lignification, cell wall structure, carbohydrate metabolism, RNA metabolism, respiration, indole acetic acid metabolism, phenol metabolism, and membranes. Most of these hypotheses are based on increased or decreased amounts of certain compounds in B deficient tissues. However, it is not likely that B is involved in such a lot of processes directly, but B should have a role in a certain fundamental function whose impairment can lead to a cascade of secondary effects in metabolism. In this regard recent studies favor plasma membranes or cell walls as the candidate for the primary action site of B.

It is frequently observed that B deficiency immediately affects the membrane transport activities. Pollard et al. (1977) reported that uptake rates of phosphorus are much lower in the root tips of B deficient as compared with sufficient bean (*Phaseolus vulgaris*) and maize (*Zea mays*) plants, and that B pretreatment for 1 h restores the uptake rate in deficient root. This is one of the most rapid response reported so far, and suggest a close relationship between the plasma membrane and B. A similar effect of B pretreatment on the uptake rates was observed for chloride and rubidium in the same study, and for glucose in cultured tobacco (*Nicotiana tabacum*) cells (Mizutani 1992). Such a general effect on membrane transport suggest that B modulates the activity of plasma membrane-bound  $H^+$ -ATPase which generates the driving force for membrane transport of various solutes. However there is no convincing evidence for the direct involvement of B in the  $H^+$ -ATPase activity. Parr and Loughman (1983) suggested that B stabilizes the structure of the plasma membrane by complexing with compounds containing *cis*-diol groups such as membrane glycoprotein or glycolipids. Cakmak et al. (1995) proposed that B has a protective effect on membrane by complexing phenolic compounds, which otherwise can be oxidized to highly toxic quinones and oxygen free radicals. These hypothesis may well explain the diverse effect of the B deficiency, as the structural and functional integrity of membrane is essential for metabolism and homeostasis of the cell. However, any complex of a membrane component and B has never been isolated to date. In addition, it is difficult to explain why only plants require B to stabilize their membrane while animals do not.

Boron-deficient plants have morphologically altered cell walls. A typical change is the irregular thickening of the walls (Spurr 1957, Kouchi and Kumazawa 1976, Hirsch



and Torrey 1980, Fischer and Hecht-Buchholz 1985). Hirsh and Torrey (1980) reported that the cell-wall thickening was observed as early as 6 h after the removal of B, and that the primary cell walls of B deficient cells are characterized by irregular depositions of vesicular aggregations near the cell wall-plasma membrane interface. Selected cells of cultured tobacco cells which have been acclimated to lower doses of B also have the swollen cell walls (Matoh et al. 1992). These suggest that B is required for the structural integrity of the cell wall. Localization of B in the cell wall, isolation of the B-polysaccharide complex (described below) and the restricted essentiality of B to cell wall-having organisms are consistent with this assumption. As the cell walls support the plasma membrane against turgor pressure, structural integrity of the cell wall should be a prerequisite for the structural and functional integrity of the membrane. It is possible that various disorder caused by B deficiency are the results of malformation in the cell wall-plasma membrane interface.

There has been relatively little work on the distribution of B within plant cells. Since the specific function of B might be associated with a certain cellular constituents, and abnormalities due to B deficiency may develop first where B should be localized, information on the distribution and the form of B within the cell would be of value.

Protoplasts prepared from suspension-cultured tobacco cells did not contain significant amounts of B (Matoh et al. 1992, Loomis and Durst 1992). To rule out a possibility that the symplastic B leaks out during protoplast preparation, Matoh et al. (1992) homogenized tobacco cells in its culture medium and assessed the carry over of the culture medium using [ $^3\text{H}$ ]- $\text{H}_2\text{O}$  and [ $^{14}\text{C}$ ]-sorbitol. The concentration of B in the supernatant could be entirely ascribed to the carry over of B from the culture medium, indicating that the symplastic B did not contribute to any increase in the B concentration of the supernatant. These data suggest that the B concentration in the symplast is negligible in cultured cells.

Intact plants contain B both in water soluble and insoluble forms, as reported by Marsh (1942) and Marsh and Shive (1941). The authors assumed that the insoluble B is functional, because the water soluble B fluctuate in amount according to the supply of B, while the insoluble B does not.  $^{11}\text{B}$ -NMR shows that the water soluble B in leaves of Swiss chard (*Beta vulgaris cicla*) occurs as free boric acid (Matoh 1997). If boric acid is present in the symplast, it would occur as esters because the symplast is rich in *cis*-diol compounds such as ribose in nucleotides and the pH is above 7. Therefore free boric acid in Swiss chard leaves must occur in the apoplast. Swiss chard protoplast does not contain significant amounts of B (Matoh 1997). These results indicate that both water soluble and insoluble B

occur in the apoplast of the plant cell.

Yamanouchi (1971) was the first to demonstrate that the amount of water-insoluble but 0.5 M HCl-soluble B is highly correlated with water- and oxalate-insoluble but 0.05 M HCl-soluble pectic polysaccharides in 52 species of higher plants. Hu and Brown (1994) also obtained a similar result that suggests the association of B with pectic polysaccharides. Yamauchi et al. (1986) presented the possibility that cell wall polysaccharides which contain B could be solubilized by hydrolysis of cell walls with pectinase enzymes. Matoh et al. (1993a) isolated and purified a particular B-polysaccharide complex from radish root cell walls. From these studies it is most likely that the physiological function of B is involved in the cell wall structure.

I started the investigations with the intracellular localization of B in cultured tobacco BY-2 cells and confirmed that B is localized in cell wall and occurs as a B-polysaccharide complex (Chapter 1). I made clear the structure of the complex (Chapter 2), and found that cooperative function of  $\text{Ca}^{2+}$  and B through the reconstitution of the complex *in vitro* (Chapter 3). Finally I proved that the complex was essential to form a network of pectic polysaccharide chains in cell walls (Chapter 4). These results indicate that B has a key role in formation of the cell wall structure of higher plants.



# Chapter 1

## Localization of Boron in Cultured Tobacco Cells

### 1.1 Introduction

Essentiality of B for growth and development of plant cells is well known, but its primary function is still unknown. The primary lesion due to B deficiency should develop as a result of the absence of B in those location where B normally occurs. Therefore, determination of the site of B in the cell should assist to identify the function of B.

Matoh et al. (1992) examined intracellular localization of B in cultured tobacco BY-2 cells and showed that a significant amount of B does not occur in the symplast. The result inclined the authors to conclude that the primary functional site for B is in the cell wall. However, based on the prompt responses of the membrane activity to B status, it has been claimed that B may bind to plasma membrane glycoproteins to stabilize the membrane structure (Parr and Loughman 1983). In this study I examined the occurrence of B in the membrane or the cell wall fraction of cultured tobacco cells, and then analyzed the form of B in those fractions.

### 1.2 Materials and methods

#### 1.2.1 Cell culture

Cultured tobacco BY-2 cells (*Nicotiana tabacum* L. cv. BY-2) were subcultured in 75 mL of a modified Linsmaier and Skoog medium with 3% sucrose and 0.2 mg liter<sup>-1</sup> 2,4-dichlorophenoxyacetic acid, pH 5.8, in a 300-mL conical flask maintained in the dark at 25°C shaken at 130 rpm (Nagata et al. 1981). The concentration of B in the medium was 1 mg B liter<sup>-1</sup> in the form of boric acid. One-week old cells were harvested by suction filtration. The cells were suspended in 20 volumes (v/w) of 0.5 mM CaCl<sub>2</sub> for 2 min and

filtered. An aliquot of the cells was taken for the B assay. The remaining cells were used in the preparation of cell wall and membrane samples.

### **1.2.2 Preparation of a cell wall fraction**

The filtered cells were suspended in four volumes (v/w) of 99.5% ethanol and filtered. The filtered cake was washed successively by suspension in 10 volumes (v/w) of 99.5% ethanol twice, 1 h each, 1 volume of chloroform-methanol mixture (2:1, v/v) overnight, and finally acetone for 1 h. The residue was allowed to air-dry in a hood and used as a cell wall preparation.

### **1.2.3 Preparation of a membrane fraction**

The washed cells (ca. 5 g) were frozen in liquid N<sub>2</sub> and homogenized with a mortar and pestle in 30 mL of HEPES-KOH buffer (pH 7.0) supplemented with 1 mM DTT and 0.5 mM EDTA with acid-washed sea sand. The homogenate was centrifuged at 2,000×g for 15 min. The supernatant was further centrifuged at 80,000×g for 1 h to pellet membranes. The pellet was washed once with water, and finally suspended in 2 mL of water.

### **1.2.4 Purification of the boron-polysaccharide complex (BPC) from cell walls**

A batch of 2 g of the cell wall preparation was suspended in 200 mL of 20 mM sodium acetate buffer (pH 4.0) containing 0.1% (w/v) Driselase (Kyowa Hakko Co., Tokyo) and incubated at 25°C for 48 h on a rotary shaker. The suspension was centrifuged (10,000×g for 15 min) and the supernatant was adjusted to pH 8.0 with a 2 M Tris solution. An aliquot was dialyzed against water to quantitate the B associated with non-dialyzable polysaccharides. The remaining of the solution was applied to a DEAE-Sepharose column (1.6×30 cm, Cl<sup>-</sup> form, Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was eluted with a 1-liter gradient of 0 to 0.5 M NaCl in the column buffer. The fractions containing B were pooled, dialyzed against water and lyophilized. The powder was dissolved in 20 mM Tris-HCl buffer (pH 8.0) supplemented with 0.1 M NaCl and applied to a Superdex 75 column (1.6×60 cm, Pharmacia) equilibrated with the same buffer. Fractions rich in B were pooled, dialyzed against water and lyophilized.

### 1.2.5 Assay methods

Boron was quantitated by the 2,4-dinitro-1,8-naphthalenediol method (Matoh et al. 1993b). Carbohydrates were assayed by the phenol-sulfuric acid method (Dubois et al. 1956) using glucose as a standard.

## 1.3 Results and discussion

More than 90% of the cellular B was localized in the cell walls of cultured tobacco BY-2 cells, when the cells were dehydrated in 80% ethanol (Table 1.1).

When fresh cells were homogenized in a buffer solution, a majority of cellular B was recovered in  $2,000\times g$  precipitate (data not presented). About 10% of the total B was recovered in the  $80,000\times g$  supernatant in a non-dialyzable form (Table 1.2). Occurrence of soluble and non-dialyzable B in the  $80,000\times g$  supernatant was first demonstrated by Skok and McIlrath (1958), and they found that this B does not fluctuate during the development of B deficiency in sunflower plants. Matoh et al. (1993a) isolated a B-polysaccharide complex from radish (*Raphanus sativus*) root cell walls, which consisted of borate and a fragment of pectic polysaccharide. As described below, tobacco cell walls also contained the complex. Therefore B in the  $80,000\times g$  supernatant may be ascribed to mechanically fragmented pectic polysaccharides which were associated with B, although the chemical structure of them could not be examined in detail because of scarcity of the sample. The  $80,000\times g$  pellet does not contain a significant amount of B. These results are consistent with those of Matoh et al. (1992) which reported that soluble B does not occur in a significant amount inside the cells, and that the protoplasts of the cells contained as much as 1.6% of the cellular B (Matoh et al. 1992). Parr and Loughman (1983) have advocated that B may work at the plasma membrane by binding to the carbohydrate moiety of membrane-associated glycoproteins. In

**Table 1.1** Recoveries of cellular boron in cell walls

	Cellular B ( $\mu\text{g g}^{-1}$ cell FW)	Wall B ( $\mu\text{g g}^{-1}$ wall)	Wall yield (g wall $\text{g}^{-1}$ cell FW)	B recovery <sup>a</sup> (%)
Exp. 1	0.52	11.4	0.043	94
Exp. 2	0.41	8.9	0.042	91
Exp. 3	0.38	9.9	0.035	91

<sup>a</sup> B recovery =  $100 \times (\text{wall B}) \times (\text{wall yield}) / (\text{cellular B})$

**Table 1.2** Distribution of B in differential centrifugation of homogenate of cultured tobacco BY-2 cells

Fractions	Boron (ng fraction <sup>-1</sup> )	
	Exp. 1 (4.17 g FW)	Exp. 2 (4.94 g FW)
Cell homogenate	2080	2250
2,000×g Supernatant	Not determined	Not determined
80% Ethanol precipitated <sup>a</sup>	101	106
80,000×g Supernatant	229	256
Dialyzed	208	213
80,000×g Precipitate	11.5	11.5

<sup>a</sup> Boron concentration was determined in different samples and the values were normalized by the fresh cell weight used.

tobacco BY-2 cells, however, such an association was not confirmed. These results suggest that in tobacco cells essentially all the B is localized in the cell wall.

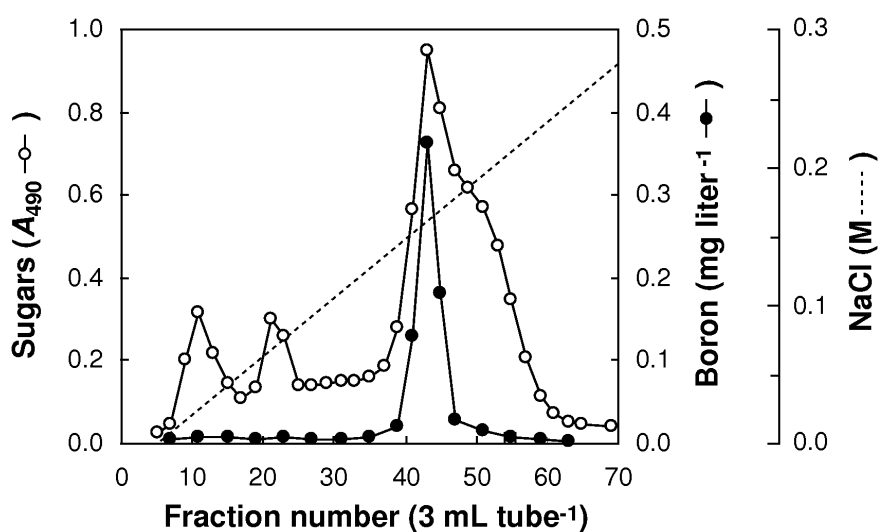
Driselase released 86.2% of the cell wall B in non-dialyzable fragments (Table 1.3). When the digest was subjected to DEAE-Sephadex chromatography, B was detected in a single peak (Fig. 1.1). Further purification with gel filtration chromatography yielded ca. 80% of the cell wall B in an apparent single peak (Table 1.3). Matoh et al. (1993a) isolated and purified a particular B-polysaccharide complex (BPC) from radish root cell walls. The BPC is solubilized from the cell wall by Driselase. It consists of an acidic polysaccharide fragment and 0.2% (w/w) of B, and has an apparent molecular weight of 7,500 (Ishigaki et al. 1993). As these character are quite similar to those of the B-containing substance isolated here, it should be the same type of BPC as that of radish root. Because of

**Table 1.3** Purification of a boron-polysaccharide complex from tobacco cell walls

	Boron (μg)	Sugar <sup>a</sup> (mg)	Boron/Sugar <sup>a</sup> (wt%)	Yield (%)	Purification (fold)
Cell walls	18.0	1,500 <sup>b</sup>	0.0012	100	
Driselase digest	15.5	503	0.0031	86.2	1.0
DEAE-Sephadex	15.1	33.7	0.045	84.2	7.1
Superdex 75	14.1	9.6	0.15	78.3	47.7

<sup>a</sup> Sugars were determined colorimetrically.

<sup>b</sup> Determined gravimetrically.



**Figure 1.1** Chromatogram of a Driselase digest of tobacco cell walls on a DEAE-Sephadex column (1×25 cm). Fractions (3 mL) were collected and assayed for total sugars (○) and B (●).

the ability of boric acid to form complexes with polyhydroxyl compounds, it seems possible that B might bind to cell wall polysaccharides at random. However, in tobacco BY-2 the BPC accounted for at least 70% of the cellular B (the cell wall contained 90% of cellular B and 80% of the cell wall B was recovered as the BPC). This result strongly suggests that the BPC is involved in the physiological function of B. The structure of the BPC is studied in the following chapter.





## **Chapter 2**

# **Structure of the Boron-Polysaccharide Complex of Radish Roots and Cultured Tobacco cells**

## **2.1 Introduction**

In cultured tobacco cells, nearly all the cellular B was localized in the walls as a sole B-polysaccharide complex (BPC). The complex was very similar to that previously isolated from radish root cell wall (Matoh et al. 1993a). As the tobacco BPC accounted for at least 70% of the cellular B, it is quite likely that this complex is directly involved in the physiological function of B. Although the association of B and cell wall polysaccharides have long been postulated (Schmucker 1933, Smith 1944, Yamanouchi 1971, Yamauchi et al. 1986), its molecular entity has not been identified. The identification and characterization of the BPC should provide the tools for analysis of the essentiality of B at a molecular level. This chapter deals with the structural analysis of the BPC.

## **2.2 Materials and methods**

### **2.2.1 Preparation of the BPC**

The BPC was prepared from radish roots as described in Chapter 1 with some modifications. Briefly, radish roots were grated and squeezed, and the macerated tissue was freeze-dried. The dried tissue was treated with Pectinase-SS (0.1% w/v; Kyowa Chemical Products Co., Ltd.) in 400 mL of 20 mM sodium acetate, pH 4.0 for 48 h at 25°C on a rotary shaker (130 rpm). The suspension was centrifuged ( $10,000\times g$  for 15 min) and the supernatant was adjusted to pH 8.0 with 2 M Tris, and then applied to a DEAE-Sepharose column (4.8×60 cm, Cl<sup>-</sup> form, Pharmacia) equilibrated with 20 mM Tris-HCl, pH 8.0. The column was

eluted with a 6-liter linear gradient of 0 to 0.5 M NaCl in the column buffer, and the fractions containing B were pooled and dialyzed. The BPC was purified by re-chromatography on the same DEAE-Sephadex column. Fractions containing B were subjected to gel-filtration using a Superdex 75 column (2.6×60 cm, Pharmacia), equilibrated with 20 mM Tris-HCl, pH 8.0 containing 0.1 M NaCl. Boron-rich fractions were pooled, dialyzed against water and lyophilized.

### **2.2.2 Partial hydrolysis of the BPC with acid**

The BPC (1 mg) was incubated in 0.1 M HCl (1 mL) for 15 min at 25°C. After neutralization with NaOH, an aliquot (100  $\mu$ L) was analyzed by size-exclusion chromatography (YMC-pack Diol-120, 300×8 mm, YMC Co. Ltd., Shimadzu 6A HPLC system). The column was equilibrated and eluted at a flow rate of 0.5 mL min<sup>-1</sup> with 50 mM sodium acetate, pH 5.2 containing 0.2 M NaCl. Saccharides were detected fluorometrically (Shimadzu RF 530 detector) after post-column labeling of their reducing termini with 2-cyanoacetamide (Honda et al. 1980) at excitation and emission wavelengths of 331 and 383 nm, respectively.

For the structural analysis of the side chains, the BPC (10 mg) was partially hydrolyzed with 0.1 M TFA (5 mL) at 40°C for 24 h (Spellman et al. 1983). The hydrolysate was adjusted to pH 9 with 1 M NH<sub>4</sub>OH and applied to a DEAE-Sephadex column (1.3×4 cm) equilibrated with 30 mM NH<sub>4</sub>HCO<sub>3</sub>, and the flow-through was collected and lyophilized. This fraction is referred to as the neutral fraction of the partial hydrolysate. Further fractionation was carried out on a Bio-Gel P-2 column (1.6×90 cm, Bio-Rad) equilibrated with 50 mM sodium acetate, pH 5.2. The two fractions (Fraction I and II) were pooled separately, desalted by passage through a column of Dowex 50W-X8 (H<sup>+</sup> form, 1×2 cm), and lyophilized.

### **2.2.3 Glycosyl residue- and glycosyl linkage-composition analyses**

Alditol acetates and trimethylsilylated methyl glycosides were prepared according to York et al. (1986) and analyzed by GLC and GLC-MS. Methylation analysis was carried out without pre-reduction as described by Harris et al. (1984) except that sodium carbanion was used. Fraction II was worked up after York et al. (1985). Partially methylated alditol acetates were analyzed by GLC and GLC-MS. Quantification was made based on GLC peak area and response factors determined empirically. When authentic standards were not

available, the effective carbon-response factors (Sweet et al. 1975) were used. The factor for the derivative of aceric acid residue was assigned to be 0.70 in glycosyl composition analysis (Thomas et al. 1989), and that for the methylated derivative of 5-linked KDO residue to be 0.87 (Sweet et al. 1975).

GLC was performed on a Shimadzu GC-15A equipped with a 15-m DB-225 capillary column (J & W Scientific). The oven temperature program was as follows: 140°C to 220°C at 2°C min<sup>-1</sup>, and 220°C for 20 min. GLC-MS was performed on a Shimadzu GC-14A-QP2000 system (electron impact ionization mode at 60 eV) equipped with a 15-m DB-225 capillary column. For the analysis of alditol acetates and trimethylsilyl ethers, the temperature program used was the same as that for GLC. For analysis of partially methylated alditol acetates, the following oven temperature program was used: 100°C to 220°C at 2°C min<sup>-1</sup>, and 220°C for 20 min.

#### **2.2.4 Determination of molecular weight**

The molecular weights of the BPC and its fragments were determined with matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker TOF-MS, Model REFLEX II equipped with a 337 nm laser) using a nominal potential of 20 kV and 2,5-dihydroxybenzoic acid as the matrix.

#### **2.2.5 <sup>1</sup>H-NMR spectroscopy**

<sup>1</sup>H-NMR spectra were recorded with a Bruker ARX-500 spectrometer operated at 500 MHz at 70°C. Samples were dissolved in deuterium oxide and chemical shifts were reported relative to internal acetone ( $\delta$  2.235).

#### **2.2.6 Boron and sugar assay**

Boron content was determined by the 2,4-dinitro-1,8-naphthalenediol method (Matoh et al. 1993b).

Total sugars were assayed by the phenol-sulfuric acid method (Dubois et al. 1956) using glucose as a standard. Uronic acids were measured by the *m*-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen 1973) using galacturonic acid as a standard. 2-Keto-3-deoxysugars were determined by the modified thiobarbituric acid method (York et al. 1985). Reducing end-groups were determined as a glucose equivalent by the 2-cyanoacetamide assay (Honda et al. 1982).

## 2.3 Results and discussion

Experiments were carried out with radish BPC throughout this chapter. Acid hydrolysis (0.1 M HCl for 15 min at 25°C) of the BPC released B and decreased its molecular weight by one half with only a slight increase (14%) in the proportion of reducing termini (Table 2.1). On size-exclusion chromatography, the elution time of the BPC delayed by the treatment from 17.8 to 19.0 min (Fig. 2.1) without yielding any detectable peaks of smaller fragments. Trace amounts of oligosaccharide were detected in the untreated BPC (22-25 min in Fig. 2.1a). Those fragments may result from degradation of the BPC during storage and did not increase significantly after the acid treatment (Fig. 2.1b). The fragment generated by the treatment was homogeneous in terms of size and charge based on its single symmetric elution peak on columns of Diol-120 and DEAE-Sepharose (data not presented). These results indicate that the acid treatment of the BPC generates two identical polysaccharide chains. The B in the radish BPC was shown by  $^{11}\text{B}$ -NMR to be present as a tetravalent 1:2 borate-diol complex (Matoh et al. 1993a) and since the reducing end-group did not increase significantly (Table 2.1), the formation of only one component with the simultaneous release of B suggests that B cross-links two identical polysaccharide chains through borate-diol ester bonds. Since the B content determined gravimetrically was 0.23% and its molecular weight is 9,900 (Table 2.1), one mol BPC contains 2.1 mol B.

The glycosyl composition of the BPC was determined by GLC and by colorimetric assays. Taking recovered sugars as 100%, the mol percent of the components was as follows: 2-*O*-methylfucose (3%), rhamnose (8%), fucose (2%), 2-*O*-methylxylose (4%), aceric acid (1%), arabinose (7%), apiose (12%), galactose (7%), 2-keto-3-deoxysugars (5%, 3-deoxy-D-*manno*-2-octulosonic acid equivalent), and uronic acid (51%, galacturonic acid equivalent).

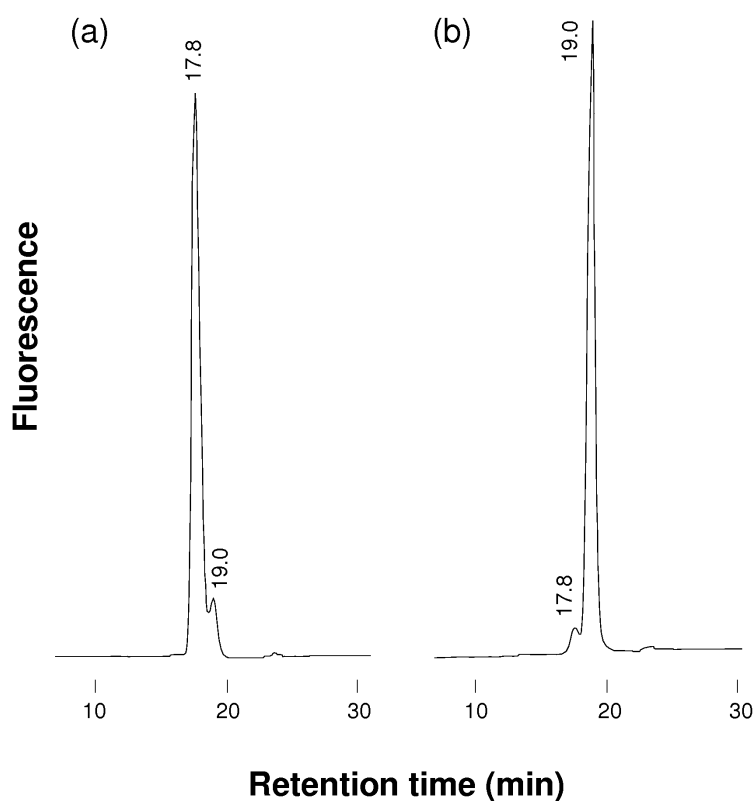
**Table 2.1** Effects of acid hydrolysis on the radish BPC

Sample	Boron/Sugar <sup>a,b</sup>	Molecular weight <sup>c</sup>	Reducing termini/ Total sugar <sup>b</sup>
	<i>wt%</i>		<i>mol%</i>
Intact	0.30	9,894	7.2
Acid-hydrolyzed	0.01	4,927	8.2

<sup>a</sup> Hydrolysate was thoroughly dialyzed against water before determining B.

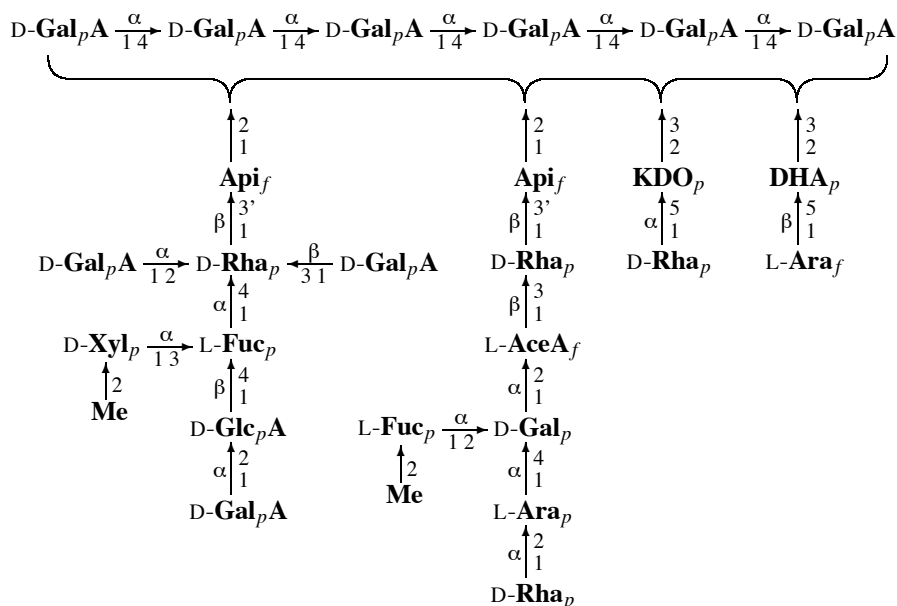
<sup>b</sup> Sugars were determined colorimetrically using glucose as standard.

<sup>c</sup> Determined by MALDI-TOF MS.



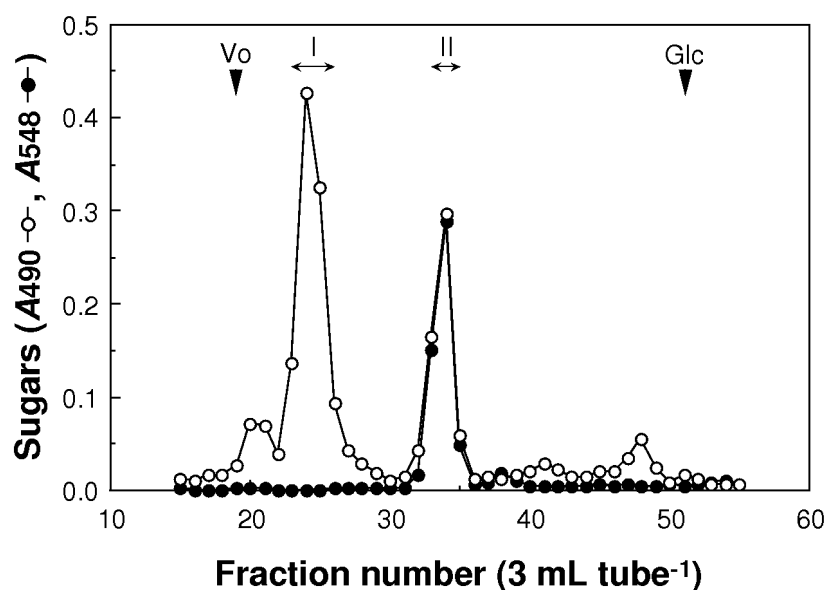
**Figure 2.1** Chromatogram for the radish BPC before and after acid hydrolysis. A batch of 1 mg BPC was incubated in 1 mL of 0.1 M HCl and a 100- $\mu$ L aliquot was subjected to size-exclusion chromatography. (a) Intact BPC (without hydrolysis); (b) BPC incubated in 0.1 M HCl at 25°C for 15 min. Saccharides were detected fluorometrically using post-column labeling of the reducing termini with 2-cyanoacetamide.

lent). The glycosyl-residue composition, except for uronic acid, is very close to those of the rhamnogalacturonan II (RG-II, Fig. 2.2) isolated from the sycamore (*Acer pseudoplatanus*) cell wall (Stevenson et al. 1988). Uronic acid percentage for the BPC is rather high compared to that for sycamore RG-II. This may be due to the different hydrolyzing-enzyme employed. Glycosyl-linkage analysis of the BPC revealed the presence of at least 11 different linkages, including 3,4-linked fucosyl, 3-linked and 2,3,4-linked rhamnosyl residues. As these component monosaccharides and glycosyl-linkages are diagnostic of RG-II (Thomas et al. 1989), further analysis was carried out following the methods developed for sycamore RG-II (Spellman et al. 1983).



**Figure 2.2** Hypothetical structure of RG-II (modified from Puvanesarajah et al. 1991). The length of the backbone and the particular residue to which each side chain is attached have not been determined. The abbreviation used are as follows: AceA, aceric acid; Api, apiose; Ara, arabinose; DHA, 3-deoxy-D-*lyxo*-2-heptulosaric acid; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; GlcA, glucuronic acid; KDO, 3-deoxy-D-*manno*-2-octulosonic acid; Rha, rhamnose; Xyl, xylose.

The neutral fraction of the partial TFA-hydrolysate consisted of two major peaks (Fraction I and II) as revealed by Bio-Gel P-2 column chromatography (Fig. 2.3). Both fractions did not contain uronic acid. Fraction II gave a positive response in the thiobarbituric acid assay which indicates the presence of 2-keto-3-deoxysugars in this fraction. The glycosyl composition of Fraction I is shown in Table 2.2. The molar ratio of each monosaccharides except for aceric acid was approximately unity. MALDI-TOF mass spectrometry of Fraction I gave a peak of  $m/z$  892.6 (average of two measurements), which may correspond to the  $[\text{M-H}_2\text{O}]^+$  ion of a hexasaccharide composed of one methylfucosyl, one rhamnosyl, one aceric acid, one arabinosyl, one apiosyl and one galactosyl residues ( $m/z$  910.8). Thus the oligosaccharide in Fraction I was considered to be a hexasaccharide. Glycosyl linkages of these residues determined by methylation analysis are listed in Table 2.3. Comparing these



**Figure 2.3** Chromatogram of the neutral fractions of the TFA hydrolysate of the BPC on a Bio-Gel P-2 column (1.6×90 cm). Fractions (3 mL) were collected and assayed for total sugars (○) and 2-keto-3-deoxysugars (●). Tube numbers 23 to 26 and 33 to 35 indicated by the double-headed arrows were pooled separately as Fractions I and II, respectively. The single-headed arrows indicate the void volume and the elution volume for glucose, respectively.

characteristics with those of the sycamore RG-II (Spellman et al. 1983), the terminal non-reducing rhamnosyl residue was not detected in the hexasaccharide derived from the radish BPC. Consistent with this finding Puvanesarajah et al. (1991) reported that RG-II isolated from Pectinol AC lacks a terminal non-reducing  $\alpha$ -L-rhamnosyl residue since Pectinol AC may contain an  $\alpha$ -L-rhamnosidase activity. The Pectinase-SS used here may also contain the same activity.

The 2-keto-3-deoxysugar in Fraction II was identified as 3-deoxy-D-*manno*-2-octul-osonic acid (KDO) by GLC-MS of its trimethylsilylated derivative (data not presented). In addition to KDO, the monosaccharide detected was rhamnose and the molar ratio was approximately 1:1 (Table 2.2). Methylation analysis showed the presence of a terminal non-reducing rhamnosyl and a 5-linked KDO residues (Table 2.3). The apparent inequality of the molar ratio was probably due to a low recovery of the KDO derivative. The  $^1\text{H}$ -NMR spectrum of Fraction II contained a signal in the anomeric region (at  $\delta$  5.1 ppm) indicating



**Table 2.2** Glycosyl composition of the oligosaccharides released from the radish BPC by TFA hydrolysis

Residue	Molar ratio
Fraction I	
2- <i>O</i> -Methylfucose	0.98 <sup>a</sup>
Rhamnose	1.00
Aceric acid	0.53 <sup>a</sup>
Arabinose	0.85
Apiose	1.09
Galactose	1.04
Fraction II	
Rhamnose	1.00
KDO	0.88 <sup>b</sup>

<sup>a</sup> Calculated using the effective carbon-response factor.

<sup>b</sup> Determined colorimetrically.

**Table 2.3** Methylation analysis of the oligosaccharides released from the radish BPC by TFA hydrolysis.

Residues	Position of <i>O</i> -CH <sub>3</sub> groups	Deduced linkage	Mol% <sup>a</sup>
Fraction I			
2- <i>O</i> -Methylfucose	2,3,4	terminal	33
Rhamnose	2,4	3-linked	16
Arabinose	2,3,4	terminal pyranose	25
Apiose	2,3	3'-linked	trace
Galactose	3,6	2,4-linked	26
Fraction II			
Rhamnose	2,3,4	terminal	82
KDO	2,4,6,7,8	5-linked	18

<sup>a</sup> Calculated using the effective carbon-response factor (see text).

**Table 2.4** The glycosyl-residue composition of the tobacco cell wall BPC

Residue	Mol% <sup>a</sup>
Rhamnose	18.4
Fucose	3.0
2- <i>O</i> -Methylfucose	2.9 <sup>b</sup>
Arabinose	10.0
Xylose	0.8
2- <i>O</i> -Methylxylose	3.3 <sup>b</sup>
Apiose	8.3
Galactose	8.4
Mannose	0.3
Aceric acid	2.2 <sup>b</sup>
2-Keto-3-deoxysugars	4.1 <sup>c</sup>
Uronic acids	38.4 <sup>d</sup>

<sup>a</sup> Taking recovered sugars as 100.

<sup>b</sup> Based on GLC peak area and effective carbon response factor.

<sup>c</sup> Determined colorimetrically as KDO equivalent.

<sup>d</sup> Determined colorimetrically as galacturonic acid equivalent.

that the rhamnosyl residue was  $\alpha$ -linked ( $J$  2.9 Hz). Therefore, the oligosaccharide in Fraction II was identified as  $\alpha$ -Rha-(1 $\rightarrow$ 5)-KDO, identical to the disaccharide derived from sycamore RG-II (York et al. 1985).

The occurrence in radish root BPC of oligosaccharide fragments that are characteristic of sycamore RG-II indicates that the polysaccharide moiety of the BPC is RG-II. Initially, sycamore RG-II had been reported to consist of ca. 60 glycosyl residues based on its elution pattern on gel filtration (Melton et al. 1986), but more recently Stevenson et al. (1988) determined RG-II to have ca. 30 glycosyl residues by end-group analysis. If the sycamore RG-II is in fact RG-II dimer cross-linked by B, the size determined by gel filtration and end-group analysis should be of dimeric and monomeric one, respectively. This may be the reason of the apparent discrepancy on the size of RG-II. Here I propose regarding to terminology that the BPC is a dimeric RG-II cross-linked by borate-diol ester bonding.

The BPC isolated from cultured tobacco BY-2 cells was also a borate-RG-II complex judging from its glycosyl residue- and glycosyl linkage-compositions (Table 2.4, 2.5). Re-

**Table 2.5** The glycosyl-linkage composition of the tobacco cell wall BPC<sup>a</sup>

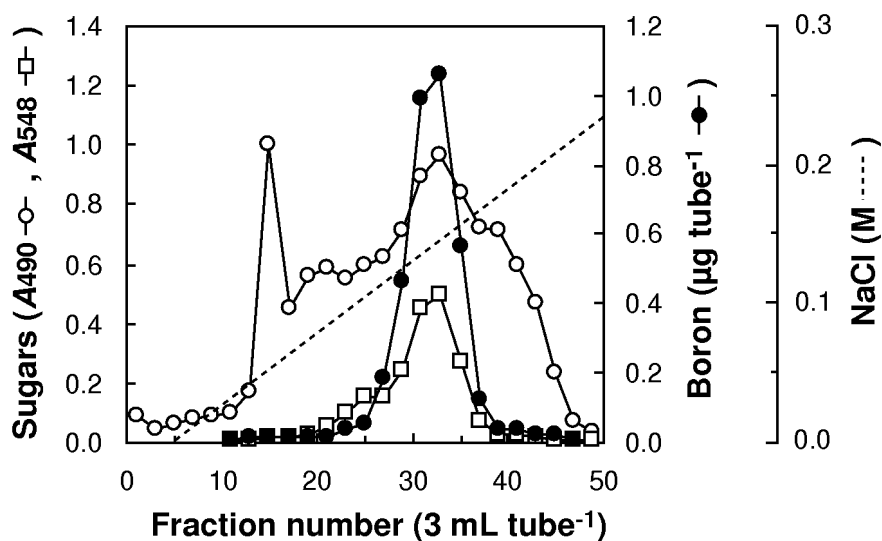
Residue	Position of O-CH <sub>3</sub> groups	Deduced linkage	Mol% <sup>b</sup>
Rhamnose	2,3,4	terminal	17.0
	2,4	3-linked	7.3
	3,4	2-linked	7.5
	none	2,3,4-linked	4.5
Fucose	2,3,4	terminal	8.9
	2	3,4-linked	7.4
Arabinose	2,3,5	terminal furanose	10.9
	4	2,3-linked pyranose	5.2
Xylose	2,3,4	terminal pyranose	7.3
Apiose	2,3	3'-linked furanose	8.4
Galactose	2,3,4,6	terminal	8.9
	3,6	2,4-linked	6.8

<sup>a</sup> Linkages of aceric acid, 2-keto-3-deoxysugars, and uronic acid residues were not analyzed.

<sup>b</sup> Calculated based on GLC peak area and effective carbon response factor, taking recovered as 100%.

cently the association of B with RG-II was confirmed in sugar beet (Ishii and Matsunaga 1996), bamboo shoot (Kaneko et al. 1997), sycamore and pea (O'Neill et al. 1996) cell walls. Match et al. (1996) examined the presence of the BPC in cell walls of 24 species of higher plants and found that in all the species examined cell wall B is judged to be associated with RG-II. They also reported that at least in 15 species RG-II is likely to be the exclusive carrier of cell wall B based on the close quantitative relationship between cell wall B and RG-II. Fern is reported to require B for growth (Lewis 1980). When the Driselase digest of *Adiantum* cell walls was fractionated on a DEAE-Sepharose column, B comigrated with 2-keto-3-deoxysugars (Fig. 2.4). These results suggest that the B-RG-II complex is ubiquitous among vascular plants, and that the B-RG-II complex is involved in the essential function of B.

In cell walls RG-II has been considered to be one region of a long-chained pectic



**Figure 2.4** Chromatogram of a Driselase digest of a fern *Adiantum capillus-veneris* leaf cell walls on a DEAE-Sepharose column (1×10 cm). A batch of 0.5 g of leaf cell walls was hydrolyzed with 0.1% (w/v) Driselase in sodium acetate buffer (pH 4.0) for 48 h, and the digest was applied to a DEAE-Sepharose column equilibrated with 20 mM Tris-HCl (pH 8.0). The polysaccharide fragments were eluted with a linear gradient of NaCl (0-0.5 M). Fractions (3 mL) were collected and assayed for total sugars (○), 2-keto-3-deoxysugars (□) and B (●).

polysaccharide (Thomas et al. 1989). If this was the case, two pectic polysaccharides could be cross-linked in the RG-II regions to form a network. Teasdale and Richards (1990) speculate that B plays a key role in contributing to cell wall strength during the expansionary phase of cell growth by participating in reversible formation of a carbohydrate gel surrounding the cellulose microfibrils. Such a function of B in cell walls was also predicted by Torssell (1956). The results obtained here substantiate their proposal. The significance of the B-RG-II complex as a cross-linker of pectic polysaccharides will be further discussed in Chapter 4.



## Chapter 3

# Reconstitution of the Borate-Rhamnogalacturonan II Complex *in Vitro*

### 3.1 Introduction

In the previous chapter the BPC has been shown to be a dimeric RG-II cross-linked by two borate-diol ester bonds. In tobacco cells the B-RG-II complex accounted for at least 70% of cellular B. Any other B-containing material could not be identified, although in plant cells there seems to be a lot of compounds with *cis*-diols besides RG-II. In this chapter I further characterize the B-RG-II complex through its reconstitution and discuss the mechanism for the formation of the complex.

### 3.2 Materials and methods

#### 3.2.1 Preparation of the monomeric RG-II and reconstitution of the B-RG-II complex

Boron was removed from the tobacco complex by incubating in 0.1 M HCl as described for the radish root BPC (Chapter 2). Monomeric RG-II was dissolved in 100 mM sodium acetate buffer (pH 4.0) at the concentration specified in the figure legend and an equimolar concentration of a boric or germanic acid solution was mixed. The molecular weight of RG-II was taken as 5,000 (Table 2.1). A 5- $\mu$ L aliquot of the mixture was applied to size-exclusion chromatography (YMC-pack Diol-120, 8 $\times$ 300 mm) with a refractive index detector (TOSOH RI-8020). The column was equilibrated and eluted with 50 mM sodium acetate buffer (pH 5.2) containing 0.2 M NaCl at a flow rate of 1 mL min<sup>-1</sup>. The proportions

of the RG-II converted to the dimer were determined by their respective peak areas.

Germanic acid was prepared by alkaline fusion of germanium dioxide with sodium carbonate followed by passing through a column of Amberlite IR-120 ( $H^+$ ) to remove sodium ion.

### **3.2.2 Analyses for divalent cations in radish B-RG-II complex**

The radish B-RG-II complex (3 mg) was dissolved in 0.3 mL of 10 mM Tris-HCl (pH 8.0) and passed through a column (1 × 1 cm) containing Chelex 100 resin (Bio-Rad) equilibrated with the same buffer. The effluent was then fractionated on a PD-10 size-exclusion column (Pharmacia) equilibrated with the same buffer. The high molecular weight fraction was collected and determined its mineral composition.

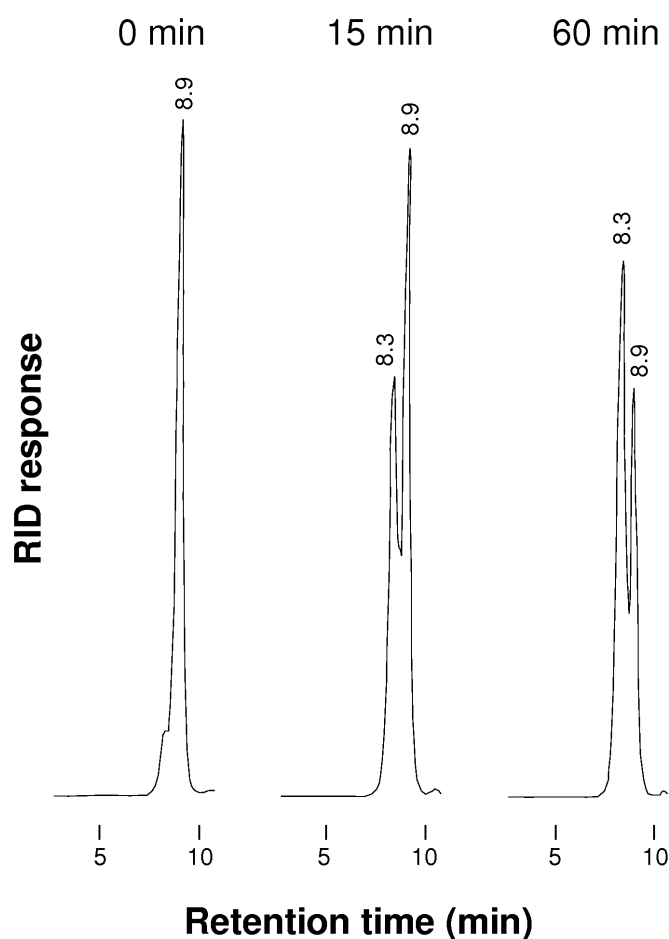
### **3.2.3 Assay methods**

$^{11}B$ -NMR spectra were recorded with a Bruker ARX-500 spectrometer operated at 160.1 MHz at 27°C and peaks were assigned according to Oi et al. (1992). Calcium was quantified by atomic absorption spectroscopy (Shimadzu AA-640). Strontium, barium or lead were quantified by atomic absorption spectroscopy (Shimadzu AA-660) utilizing a graphite furnace atomizer (Shimadzu GFA-4A).

## **3.3 Results**

### **3.3.1 Reconstitution of B-RG-II complex *in vitro***

Monomeric RG-II of tobacco eluted from the YMC Diol-120 column at a retention time of 8.9 min (Fig. 3.1). Incubation of the monomeric RG-II at a final concentration of 5 mM with 5 mM boric acid at pH 4.0 yielded a polysaccharide peak eluted at 8.3 min, which is the position of the native B-RG-II complex (Fig. 3.1). The polysaccharide was associated with B at nearly the same ratio as that of the native B-RG-II complex (data not presented). A  $^{11}B$ -NMR spectrum of the mixture gave a signal at 9.6 ppm (Fig. 3.2), which corresponds to the signal of the native B-RG-II complex (Matoh et al. 1993a). These results suggest that the B-RG-II complex was reconstituted spontaneously *in vitro*. Fig. 3.3 shows a time-dependent increase in mol% of the dimeric RG-II. More prompt reconstitution was observed when higher concentrations of RG-II were supplied. Increasing concentration of boric acid did not accelerate the reaction as effectively as that of RG-II (data not shown),

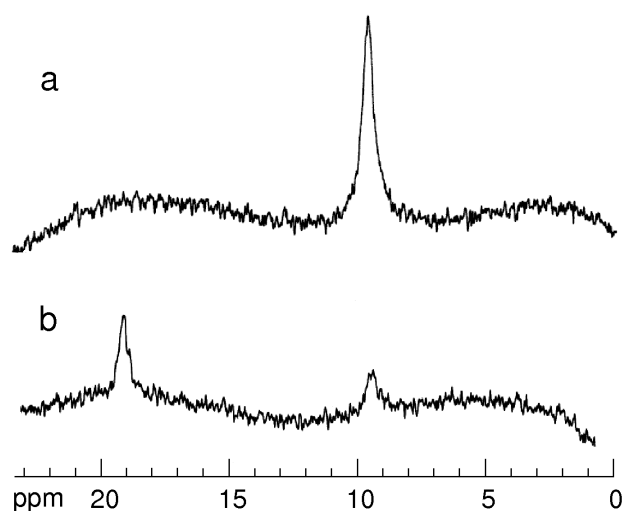


**Figure 3.1** Time course of the reconstitution of the B-dimeric RG-II. The monomeric RG-II and B were incubated in 40  $\mu\text{L}$  of 50 mM sodium acetate buffer (pH 4.0) at a final concentration of 5 mM each. A 5- $\mu\text{L}$  aliquot was subjected to size-exclusion chromatography at the time indicated.

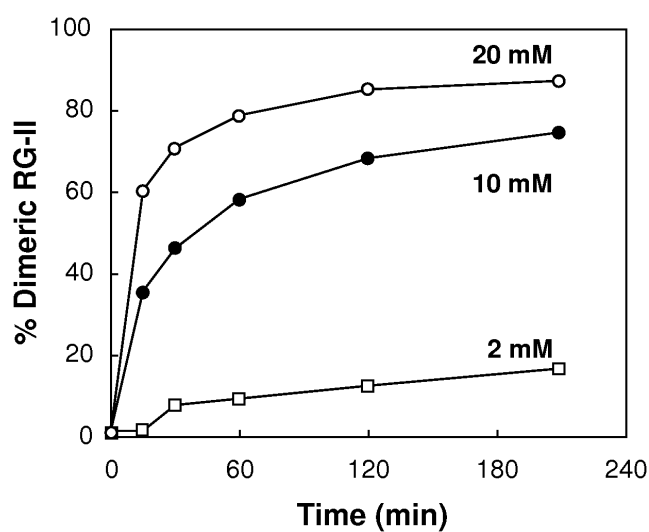
suggesting that the association of two RG-II chains determined the reaction rate. The optimum pH for the reconstitution was around 4 and the rate decreased when the pH was below 3 and above 5 (data not shown).

When germanic acid was mixed with the monomeric RG-II in place of boric acid, a new peak in the size-exclusion chromatography appeared at the same position of the B-RG-II complex (data not shown), suggesting that the dimeric RG-II was formed with germanic acid. The rate of the dimer formation was faster with germanic acid than boric acid, while

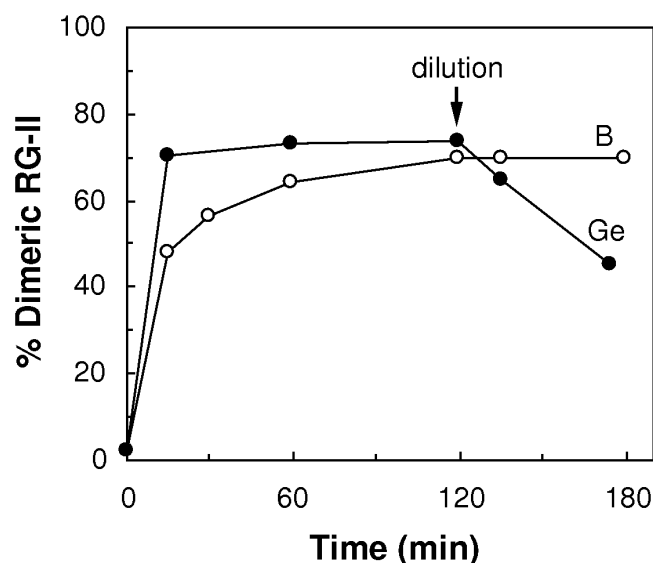




**Figure 3.2**  $^{11}\text{B}$ -NMR spectra of (a) the tobacco BPC (5 mM) and (b) the reconstituted B-dimeric RG-II. Tobacco monomeric RG-II and boric acid each at a final concentration of 20 mM were mixed and incubated for 3 h at a room temperature. The chemical shift was expressed relative to external boron trifluoride etherate.



**Figure 3.3** The rate of the formation of B-dimeric RG-II complex. Equimolar concentrations of the monomeric RG-II and boric acid were incubated in 50 mM sodium acetate buffer (pH 4.0) at a final concentration of 2 mM ( $\square$ ), 10 mM ( $\bullet$ ) or 20 mM ( $\circ$ ). At time intervals a 5- $\mu\text{L}$  aliquot was subjected to size-exclusion chromatography equipped with refractive index detector. The rate of the dimeric RG-II formation was expressed by the change in the mol% of RG-II converted into the dimer.

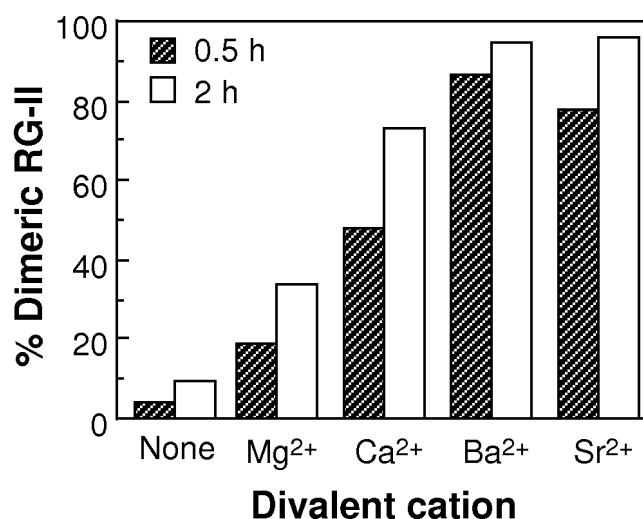


**Figure 3.4** Formation of the dimeric RG-II with boric and germanic acid. The monomeric RG-II and boric or germanic acid were incubated in 50 mM sodium acetate buffer (pH 4.0) at a final concentration of 5 mM each. At 120 min (indicated by arrow) the mixture was diluted 5-fold with 50 mM sodium acetate buffer (pH 4.0). The ordinate indicates the mol% of RG-II converted into the dimer.

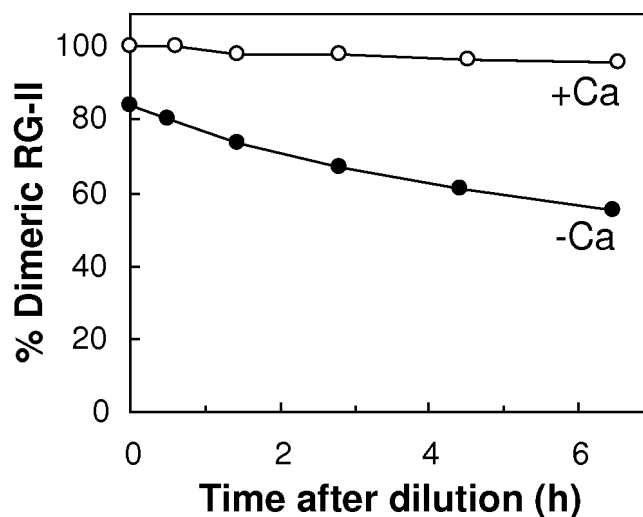
it dissociated immediately upon dilution (Fig. 3.4).

During the course of this study, O'Neill et al. (1996) reported that B-RG-II complex isolated from the walls of sycamore cells and pea stems contains divalent metal cations, such as  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Sr^{2+}$ , and  $Pb^{2+}$ . These cations and  $Zn^{2+}$  are also present in B-RG-II complex isolated from beet and bamboo (Matsunaga et al. 1997). B-RG-II complex isolated from red wine also contains  $Ca^{2+}$  (Pellerin et al. 1996). The radish B-RG-II complex contained  $Ca^{2+}$ ,  $Sr^{2+}$ ,  $Ba^{2+}$ , and  $Pb^{2+}$  at molar ratios to B of 1.2, 0.1, 0.01, and 0.04, respectively (Table 3.1). The occurrence of  $Ca^{2+}$  at an equimolar concentration to B was also confirmed in the B-RG-II complex isolated from celery, cultured tobacco cells, and cabbage (data not presented).

O'Neill et al. (1996) also reported that the supplement of divalent cations such as  $Sr^{2+}$ ,  $Ba^{2+}$ , and  $Pb^{2+}$  stimulates B-RG-II complex formation *in vitro*. Following their report I confirmed that  $Sr^{2+}$ ,  $Ba^{2+}$ , and  $Pb^{2+}$  promoted the reaction and that  $Ca^{2+}$  was also effective (Fig. 3.5). The complex reconstituted in the presence of  $Ca^{2+}$  contained 1.1 to 1.3 mol of



**Figure 3.5** Effect of divalent cations on the rate of reconstitution of the B-RG-II. Monomeric RG-II (1 mM) was incubated in 50 mM sodium acetate buffer (pH 4.0) with 10 mM H<sub>3</sub>BO<sub>3</sub> and 10 mM metal cations. The metal cations were added as chloride salts. At 0.5 and 2 h, a 10- $\mu$ L aliquot was subjected to size-exclusion HPLC with a refractive index detector. The ordinate indicates the mol% of RG-II converted into the dimer.



**Figure 3.6** Effects of Ca<sup>2+</sup> on the stability of the reconstituted B-RG-II complex. Boric acid (50 mM) and monomeric RG-II (5 mM) were incubated in 50 mM sodium acetate buffer (pH 4.0) with (○) or without (●) 50 mM CaCl<sub>2</sub>. After 20 h, the reaction mixture was diluted 10 times with the acetate buffer and free boric acid and Ca<sup>2+</sup> was removed with Amberlite IRA-743 resin (SO<sub>4</sub><sup>2-</sup> form) and Dowex 50W-X8 resin (Na<sup>+</sup> form), respectively. At intervals a 10- $\mu$ L aliquot was subjected to size-exclusion HPLC with a refractive index detector. The ordinate indicates the mol% of RG-II converted into the dimer.

**Table 3.1** Mineral contents of the radish B-RG-II complex<sup>a</sup>

Element	Treatment	
	Native <sup>a</sup>	CDTA <sup>b</sup>
	<i>mol mol<sup>-1</sup> complex</i>	
B	1.8 (1.0) <sup>c</sup>	0.78
Ca <sup>2+</sup>	2.2 (1.2)	0.05
Sr <sup>2+</sup>	0.20 (0.1)	- <sup>d</sup>
Pb <sup>2+</sup>	0.06 (0.04)	- <sup>d</sup>
Ba <sup>2+</sup>	0.03 (0.01)	- <sup>d</sup>

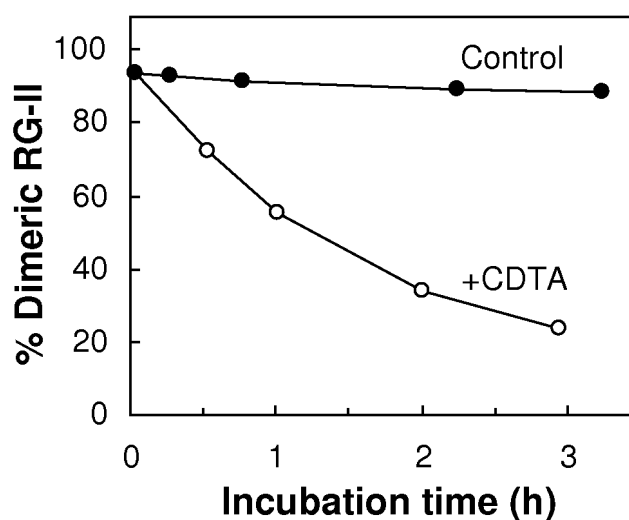
<sup>a</sup> Native radish B-RG-II complex was treated with the Chelex resin before mineral analyses.

<sup>b</sup> Radish B-RG-II complex was incubated with 50 mM CDTA in sodium acetate buffer (pH 6.5) for 3 h and then treated with the Chelex resin before analyses.

<sup>c</sup> Values in parentheses designate mol ratios to B.

<sup>d</sup> -, Not determined.

Ca<sup>2+</sup> per mol of B (data not presented). Divalent cations alone did not bring about dimer formation (data not shown), as O'Neill et al. (1996) demonstrated. The B-RG-II complex generated in the absence of Ca<sup>2+</sup> gradually decomposed into a monomer when the RG-II concentration in the reconstitution solution was reduced by dilution and free B and free Ca<sup>2+</sup> were removed by ion-exchange resins (Fig. 3.6). However, the B-RG-II complex reconstituted in the presence of Ca<sup>2+</sup> was stable under the same conditions. Removal of the Ca<sup>2+</sup> from the native B-RG-II with CDTA induced release of B (Table 3.1) and hence decay of the complex (Fig. 3.7). The spontaneous decomposition of B-RG-II complex generated without Ca<sup>2+</sup> (Fig. 3.6) suggests that removing Ca<sup>2+</sup> from the dimer renders the dimer unstable and allows it to decompose spontaneously. These results are compatible with the notion that Ca<sup>2+</sup> is a native constituents of the B-RG-II complex.



**Figure 3.7** Decomposition of the native B-RG-II complex with CDTA. Radish B-RG-II complex (1 mM) was incubated with 50 mM CDTA (pH 6.5, ○), or 10 mM MES-NaOH (pH 6.5, ●). The ordinate indicates the mol% of RG-II converted into the dimer.

### 3.4 Discussion

The B-RG-II complex was reconstituted *in vitro* simply by mixing the monomeric RG-II with boric acid. The maximum formation of the dimer occurred around pH 4 (in this study) and between pH 3 and 3.4 (O'Neill et al. 1996). O'Neill et al. (1996) suggested that the apiosyl residues of RG-II are the binding sites for B. Apiose has been proposed to be the best candidate for fixing B among sugars (Loomis and Durst 1992). However, even with apiose the borate ester is more stable under neutral to alkaline pH (Loomis and Durst 1992). This apparent discrepancy may be explained by the anionic character of RG-II. As the backbone of RG-II is a polygalacturonic acid, the negative charge of the fragments increases as pH rises and may repel the two fragments from each other and this may hinder the dimer formation. Maximum dimer formation under  $\text{pH} \leq 4$  is likely to support the assumption, since the  $\text{pK}_a$  of pectic polysaccharide is in the range of 3.5-4.0 (Jarvis 1984) and a protonated form will prevail at this low pH.

The reconstitution with B and monomeric RG-II only required a higher concentration of RG-II (Fig. 3.3), and the reconstituted complex was unstable (Fig. 3.6). It is now sug-

gested that  $\text{Ca}^{2+}$  is a constituent of the naturally occurring B-RG-II complex and that  $\text{Ca}^{2+}$  accelerates the rate of dimer formation *in vitro*. O'Neill et al. (1996) reported that low concentration (0.5 mM) of  $\text{Ca}^{2+}$  does not stimulate dimer formation effectively, that  $\text{Mg}^{2+}$  and  $\text{Cu}^{2+}$  are inhibitory, and that monovalent cations have no discernible effect. However in this study higher concentrations (10 mM) of  $\text{Ca}^{2+}$  stimulated dimer formation, and  $\text{Mg}^{2+}$  and  $\text{Cu}^{2+}$  were also effective, although the effects were small. It was also observed that high concentrations (ca. 100 mM) of monovalent cations such as  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{NH}_4^+$  also promoted the reaction to a certain extent (data not shown). The apparent discrepancies regarding the effects of  $\text{Ca}^{2+}$  and other cations may be due to the different experimental conditions. In the experiments reported here monomeric RG-II and boric acid were mixed at a final concentration of 1 mM and 10 mM, respectively, and divalent cations were added at 10 mM. These concentrations are higher than those used by O'Neill et al. (0.5 mM monomeric RG-II, 1.2 mM boric acid and 0.5 mM divalent cation). Thus, the reactivity and effectiveness of coexisting cations may vary significantly according to the concentrations used.

The effect of monovalent cations on the rate of the reconstitution may be due to the masking of the negative charge due to galacturonic acid residues of the RG-II backbone. Even though some part of the effect of divalent metal ions may be due to their cationic nature, the effect of divalent cations such as  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Pb}^{2+}$  and  $\text{Ca}^{2+}$  are superior to the monovalent ones. The dimer and the monomer is under equilibrium in the reconstitution solution. The divalent metal cations may stabilize the formed dimeric B-RG-II complex and hence shift the equilibrium in favor of the dimer. O'Neill et al. (1996) suggested that divalent metal cations may form metal coordination complexes with B-RG-II complex, since the cations are not removed by treatment with Chelex resin, and the cations are not bound to monomeric RG-II. Smaller cations such as  $\text{Ca}^{2+}$  may not interact strongly enough with the ligands on the two chains in order to stabilize the complex, however, higher concentrations may compensate for their lower affinity. Calcium ions in the naturally occurring and reconstituted B-RG-II complex are not removed by the Chelex and the Dowex 50 resins. Therefore, the  $\text{Ca}^{2+}$  may not only bind to the carboxyl groups ionically but may also form coordination bonds as has been suggested for  $\text{Sr}^{2+}$ . Van Duin et al. (1987) reported that  $\text{Ca}^{2+}$  forms coordination complexes with borate diesters of polyhydroxy carboxylates. In their model the  $\text{Ca}^{2+}$  is considered to interact with borate anion directly. However, O'Neill et al. (1996) suggested that the metal cations in B-RG-II complex do not interact with borate anion, as the cations do not affect the  $^{11}\text{B}$ -NMR spectrum of the complex. Some

carboxyl or hydroxyl groups along the RG-II sugar moiety may provide sites which are favorable to coordinate the cations.

The conditions that promote dimer formation in cell wall are not known. However, we suggest that  $\text{Ca}^{2+}$  stabilizes the B-RG-II complex *in vivo* based on the following considerations. The concentration of water-soluble  $\text{Ca}^{2+}$  in root tip cell walls is approximately 2.5 mM (Bjorkman and Cleland 1991), while the content of  $\text{Sr}^{2+}$  in angiosperm is three orders lower than that of  $\text{Ca}^{2+}$  (Bowen 1966), even though the effect of  $\text{Ca}^{2+}$  on the stabilization is not as high as  $\text{Sr}^{2+}$ ;  $\text{Ca}^{2+}$  is more abundant in soil than  $\text{Sr}^{2+}$ ; the nutritional requirement of  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Pb}^{2+}$  has not been reported unequivocally for higher plants (Bowen 1966), except for the substitution effect of  $\text{Sr}^{2+}$  under  $\text{Ca}^{2+}$ -deficient conditions (Da Silva 1962); finally,  $\text{Sr}^{2+}$ ,  $\text{Pb}^{2+}$  and  $\text{Ba}^{2+}$  account for only 0.15 mol per mol B in the native radish B-RG-II (Table 3.1) and less than 0.21 mol per mol B-RG II (O'Neill et al. 1996), in contrast to the higher content of  $\text{Ca}^{2+}$  (1.1 mol to 1 mol B) in native B-RG-II.

In intact cell walls, other factors may affect the readiness of the dimer formation. The rate of dimer formation seemed to be governed by the frequency of the association of two RG-II chains. As the RG-II regions incorporated in long-chained pectic polysaccharides do not diffuse as freely as the RG-II fragment, the alignment of the polysaccharides should determine the probability of association of two RG-II regions. Methyl-esterification of the carboxyl groups in the galacturonic acid residues within or around the RG-II region may decrease the negative charge of the RG-II regions, and hence promote the reaction by reducing the electrostatic repulsion between the regions. Although the borate ester linkage itself can be formed without any enzyme, a possibility could not be ruled out that some proteinous factors such as pectin methylesterase indirectly control the dimer formation by modifying the accessibility of the RG-II regions to each other.

Germanic acid was also able to form the dimeric RG-II although the complex seemed less stable than the B-RG-II complex (Fig. 3.4). Skok (1957) found that germanic acid can substitute for B to some extent in the growth of B-deprived sunflower plants. Loomis and Durst (1992) reported that in cultured carrot cells the minimum requirement for Ge is 10 times higher than B. They speculated that it was because germanic acid coordinates to its binding site less tightly than boric acid does. The ability of germanic acid to form a dimeric RG-II complex *in vitro* may explain the substitution effect of Ge when B is deprived from culture media. This, in turn, suggests that the formation of the cross-linkage of pectic chains is the essential function of B.

## Chapter 4

# Physiological Function of the Borate-Rhamnogalacturonan II Complex

### 4.1 Introduction

In cell walls, it is highly likely that the RG-II is linked with polygalacturonic acid and rhamnogalacturonan I to form long-chained pectic polysaccharides (Thomas et al. 1989). If this is the case, cross-linking in the RG-II region will result in formation of a supramolecular network of the pectic polysaccharides and may modify the physical property of the cell wall. This may be the primary function of B in higher plants. However, it has been believed that pectic polysaccharides are cross-linked primarily by  $\text{Ca}^{2+}$  through coordination bonding at the polygalacturonic acid region (egg-box model, Jarvis 1984). In addition, RG-II has not been considered as a structural polymer because of its scarcity in cell walls. Rather its complex structure led researchers to speculate RG-II as a signal molecule (Carpita and Gibeaut 1993). In this chapter I have evaluated the contribution of the B-RG-II complex as a constituent of pectic network by sequential extraction of pectic polysaccharides from radish root cell walls.

### 4.2 Materials and methods

#### 4.2.1 Sequential extraction from radish root cell walls

Radish (*Raphanus sativus* L. cv. Aokubi-daikon) roots purchased at a local market were homogenized in ice-cold water with a kitchen blender, and the homogenate was filtered under suction. The filtered cake was washed three times by suspension in 20 volumes (v/w) of ice-cold water, and then used as a crude cell wall preparation. A 5-g (wet weight) aliquot



of the crude cell wall was put into a column (3.2 cm diameter), and washed with 750 mL of a 1.5% (w/v) SDS solution buffered with 10 mM MES-NaOH (pH 6.5), and then 200 mL of water at a flow rate of 4 mL min<sup>-1</sup>. The SDS-washed cell wall was treated for 18 h at 25°C with 100 mL of 50 mM CDTA (pH 6.5). The residue was further extracted for 18 h at 4°C and then 2 h at 25°C with 2 mM CDTA dissolved in 50 mM Na<sub>2</sub>CO<sub>3</sub>. Aliquot was taken at each step and it was washed with distilled water and freeze-dried before the uronic acids were determined. The SDS-washed cell wall was digested with Driselase and the digest was chromatographed on a column (1×20 cm) of DEAE-Sepharose (Cl<sup>-</sup> form, Pharmacia) as described in Chapter 1.

#### 4.2.2 Assay methods

Boron was quantified by the chromotropic acid method (Matoh et al. 1997). Calcium was quantified by atomic absorption spectroscopy (Shimadzu AA-640). Pectic polysaccharides in cell walls or extracted residues were determined as galacturonic acid equivalent after Ahmed and Labavitch (1977). Wet samples were lyophilized prior to dissolution in H<sub>2</sub>SO<sub>4</sub>. Total sugar contents were assayed by the phenol-sulfuric acid method (Dubois et al. 1956) using glucose as a standard. 2-Keto-3-deoxysugars were determined by the modified thio-barbituric acid method (York et al. 1985).

### 4.3 Results

To evaluate the contribution of the B-RG-II complex as a component of the pectic network, sequential extraction of pectic polysaccharides was carried out. In this study, conventional methods for preparing cell wall using organic solvents were avoided, because dehydration may alter the solubility of the polysaccharides, and a 1.5% (w/v) SDS solution, pH 6.5 with 10 mM MES-NaOH, was used to reduce autolytic activities.

The SDS washing removed 98% of the Ca<sup>2+</sup> from the homogenized root (Table 4.1), probably because of an ion-exchange action of Na<sup>+</sup> in the SDS solution. Concomitantly, the treatment removed 17% of B and 22% of pectic polysaccharides (Table 4.1). This result is consistent with the report by Jarvis (1982), which demonstrated that during preparation of the cress, cucumber and celery cell walls with 0.5% SDS, negligible amounts of pectic polysaccharide are lost. A molar ratio of Ca<sup>2+</sup> to B of the SDS-washed cell walls was less than 1.5, which was in the same range as that of the purified B-RG-II complex (Table 3.1). Extraction of the SDS-washed cell walls with CDTA removed all of the remaining Ca<sup>2+</sup> and

**Table 4.1** Sequential extraction of  $\text{Ca}^{2+}$ , B and pectic polysaccharides from radish root cell walls

Sample	$\text{Ca}^{2+}$	B	Pectic poly-saccharides <sup>a</sup>	2-Keto-3-deoxysugars <sup>b</sup>
Crude cell walls	100(13) <sup>c</sup>	100(0.25)	100 (25)	100 (1.0)
SDS-treated cell walls	2	83	$78 \pm 0.4$	$90 \pm 2.8$
SDS- and CDTA-treated cell walls	0	5	$29 \pm 2.1$	$34 \pm 0.7$
SDS-, CDTA- and alkaline-CDTA-treated cell walls	— <sup>d</sup>	—	$14 \pm 0.5$	$29 \pm 2.8$

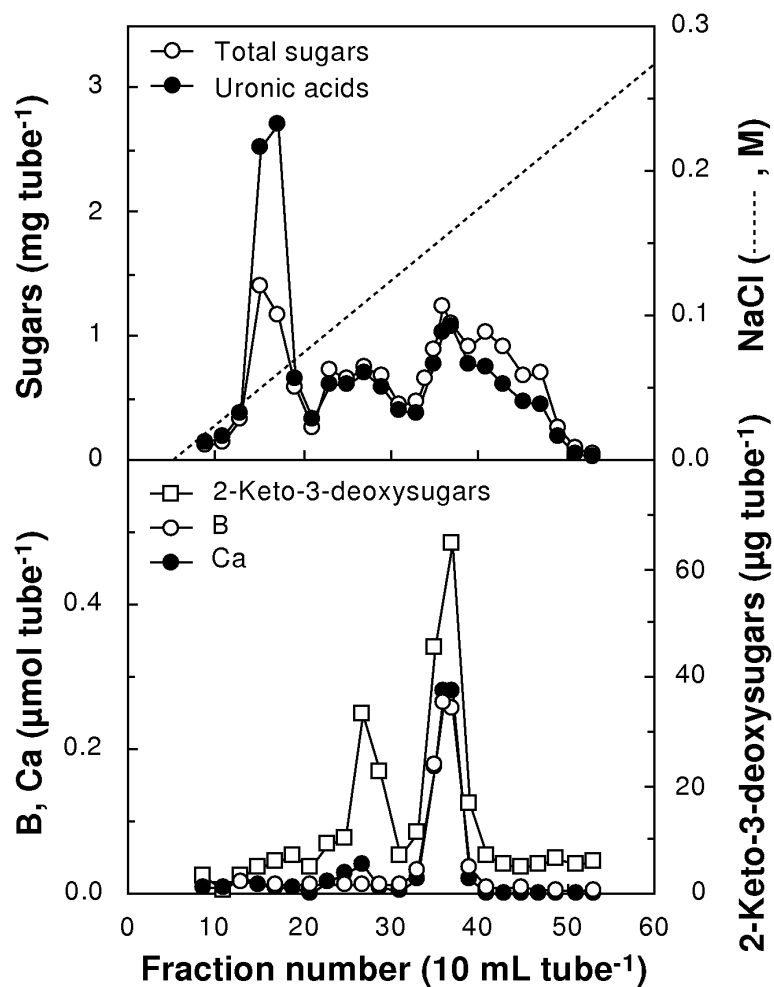
<sup>a</sup> Determined by *m*-hydroxydiphenyl method using galacturonic acid as a standard.

<sup>b</sup> Determined by modified thiobarbituric acid method using 3-deoxy-D-manno-2-oxulosonic acid as a standard.

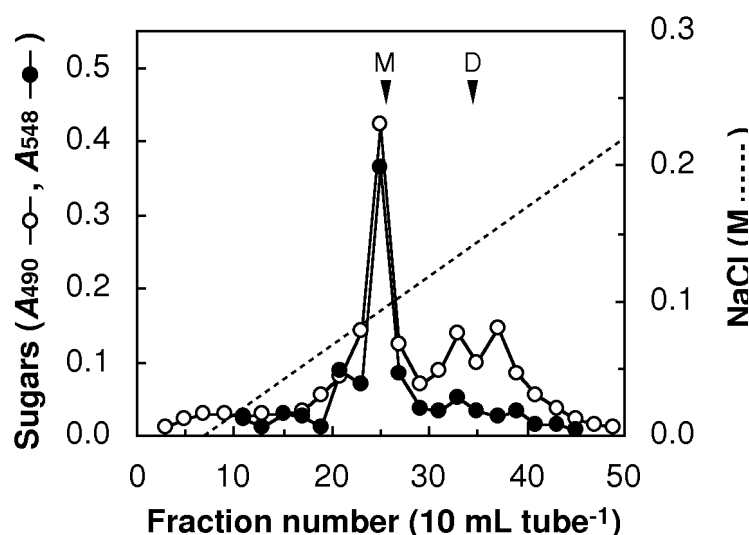
<sup>c</sup> Values in the parentheses are  $\mu\text{mol Ca}^{2+}$ ,  $\mu\text{mol B}$  and  $\mu\text{mol 2-keto-3-deoxysugars g}^{-1}$  crude cell walls, and  $\text{mg uronic acid g}^{-1}$  crude cell wall.

<sup>d</sup> —, Not determined.

78% of B, as well as 49% of pectic polysaccharides (Table 4.1). After the CDTA treatment, the walls retained a portion of 29% of pectic polysaccharides, and one-half of the remaining pectic polysaccharides were released with the alkaline-CDTA treatment. SDS-, CDTA-, and alkaline-CDTA solubilized pectic polysaccharides all contain RG-II, because 2-keto-3-deoxysugars, which are the diagnostic monosaccharides for RG-II (York et al. 1985), also decreased concomitantly (Table 4.1). RG-II may occur mainly in the CDTA fraction, judging from the contents of 2-keto-3-deoxysugars in the three fractions. Occurrence of the RG-II region in both CDTA- and alkaline-CDTA-solubilized pectic polysaccharides has been also demonstrated using an antibody toward RG-II (Matoh et al. 1998). The SDS-washed cell walls were digested with Driselase and the digest was fractionated on DEAE-Sepharose. As presented in Fig. 4.1,  $\text{Ca}^{2+}$  comigrated with B and 2-keto-3-deoxysugars.  $\text{Ca}^{2+}$  in the RG-II peak accounted for 80% of the  $\text{Ca}^{2+}$  present in the SDS-washed cell walls. These results suggest that almost all of the  $\text{Ca}^{2+}$  retained in the SDS-washed cell walls occurs in the RG-II region of the pectic polysaccharides. The CDTA treatment eliminated not only  $\text{Ca}^{2+}$  but also B from the purified RG-II complex (Table 3.1), which may have resulted in decomposition of the complex into monomeric RG-II. The  $\text{Ca}^{2+}$ -B cross-linking in the RG-II region in the SDS-washed cell wall may also be decomposed by the CDTA extraction, because Driselase digestion of the CDTA extract of the SDS-washed cell walls



**Figure 4.1** Chromatogram of a Driselase digest of the SDS-washed cell walls on DEAE-Sepharose. About 10 g of the SDS-washed cell walls was hydrolyzed with Driselase and the digest was chromatographed on a DEAE-Sepharose column ( $1.6 \times 17$  cm), which had been equilibrated with 20 mM Tris-HCl (pH 8.0). The pectic fragments were eluted with a 1-liter gradient of 0 to 0.5 M NaCl in 20 mM Tris-HCl (pH 8.0).



**Figure 4.2** Chromatogram of a Driselase digest of the CDTA extract of the SDS-washed residues on DEAE-Sepharose. About 15 g of the SDS-washed cell walls was extracted with 100 mL of 50 mM CDTA (pH 6.5) at 25°C for 18 h. The extract was dialyzed against distilled water and subjected to hydrolysis with Driselase. The column (1.6×17 cm) was equilibrated with 20 mM Tris-HCl (pH 8.0) and eluted with a 1-liter gradient of 0 to 0.5 M NaCl in 20 mM Tris-HCl (pH 8.0). Fractions (10 mL) were collected and assayed for total sugars (○) and 2-keto-3-deoxysugars (●). The arrowheads indicate the elution position of the radish monomeric (M) and dimeric (D) RG-II on the same column.

yielded only monomeric RG-II, which was free of B (Fig. 4.2). The pectic polysaccharides that are not released with SDS but are released with CDTA are probably retained in cell walls through Ca<sup>2+</sup>-B cross-linking at the RG-II regions.

## 4.4 Discussion

In cell walls, pectic polysaccharides are cross-linked by  $\text{Ca}^{2+}$  bridges, phenolic coupling, galacturonate ester bonds (Fry 1986), and borate-diester bonds (in this study). Jarvis (1982) demonstrated that CDTA effectively solubilizes pectic polysaccharides from cell walls. The chelator-soluble pectic polysaccharides has been considered to be retained in cell wall simply by  $\text{Ca}^{2+}$  bridges in the polygalacturonic acid (PGA) region. However, as CDTA breaks not only  $\text{Ca}^{2+}$ -PGA bridges but also B-RG-II complex (Table 3.1, Fig. 3.7), it is possible that the released polysaccharides have been retained by the B-RG-II cross-linking. The sequential extraction experiment demonstrated that B-RG-II complex cross-links at least 49% of pectic polysaccharides and are strong enough to retain pectic polysaccharides within the cell wall. Actually the linkage occurs more because the CDTA-extracted residue still contained 2-keto-3-deoxysugars (Table 4.1). Therefore it is likely that the B-RG-II cross-linking contribute substantially to the pectic network in cell walls.

Consideration into the difference of the  $\text{Ca}^{2+}$ -B-RG-II and  $\text{Ca}^{2+}$ -PGA cross-linking between pectic polysaccharide chains may provide a clue to elucidate the physiological significance of B. Talbott and Ray (1992) estimated that PGA region makes up about 82% of polyuronide backbone of pectic polysaccharides. This indicates that the possible binding site for B occurs much less frequently than that for  $\text{Ca}^{2+}$ . In addition, B binds only to the specific residue (possibly apiosyl) of the RG-II region. Although formation of the  $\text{Ca}^{2+}$ -PGA bridges may be controlled by the esterification/deesterification of the carboxyl groups on the uronic residues (Fry 1986), the association of  $\text{Ca}^{2+}$  to the unesterified block could be rather nonspecific. Therefore  $\text{Ca}^{2+}$ -B-RG-II linkage should be much more site-specific than the  $\text{Ca}^{2+}$ -PGA bridges are.

During the course of these experiments it is observed that the packed volume of cell walls increased 2-fold after SDS-washing, and that the change was reversed by incubation with 50 mM  $\text{CaCl}_2$  (data not presented). This suggest that the SDS-extractable  $\text{Ca}^{2+}$ , which accounts for the 98% of the cell wall  $\text{Ca}^{2+}$  and is probably associated with the polygalacturonic acid regions, controls the physicochemical properties of the cell wall. Demarty et al. (1984) mentioned the swelling of the cell walls by replacement of cell-wall  $\text{Ca}^{2+}$  to  $\text{Na}^+$ . They speculated the mechanism as follows: because the affinity of  $\text{Na}^+$  to carboxylic groups is much less than  $\text{Ca}^{2+}$ , the substitution of  $\text{Ca}^{2+}$  to  $\text{Na}^+$  increases the amount of fixed negative charge in cell wall and produces an excess of cations within the cell wall compared to the external solution; then the cell walls absorb water according to the osmotic

pressure and swells. Monovalent cations could not form the interchain cross-linking of pectic polysaccharides, which may be another reason for the swelling. It is frequently observed that B-deficiency causes a thickening of the cell wall (Spurr 1957, Kouchi and Kumazawa 1976, Hirsch and Torrey 1980). Matoh et al. (2000) reported that tobacco cells acclimated to low level of B also have a thickened cell wall, although the cell-wall  $\text{Ca}^{2+}$  content was 78% of that of the control cells. Mühling et al. (1998) found that the apoplastic concentration of free  $\text{Ca}^{2+}$  is increased by B deficiency, and the change are reversed by re-supply of B. These results suggest that B deficiency may hinder the binding of  $\text{Ca}^{2+}$  to the PGA regions. I assume that B may align the pectic polysaccharides correctly by cross-linking them at the RG-II region, and the alignment is required for  $\text{Ca}^{2+}$  to form the interchain cross-linking at the PGA regions.

What is clarified in this study is that B cross-links two pectic polysaccharide chains at the RG-II region and  $\text{Ca}^{2+}$  strengthen the linkage. However, this does not directly elucidate the mechanism underlying the abrupt cease of elongation of roots (Kouchi and Kumazawa 1975) and pollen tubes (Schmucker 1933), and the immediate death of cultured cells (Matoh et al. 1992) due to the withdrawal of B. There are many reports that B deficiency does not affect directly to the cell wall polysaccharide composition or synthesis of new cell wall materials (Slack and Whittington 1964, Goldbach and Amberger 1986). Therefore, as Loomis and Durst (1992) suggested, it is likely that B deficiency damages the cell by disturbing the structural organization of the cell wall. This may be caused by the absence of B from the RG-II regions. Reconstitution experiment has shown that B-RG-II complex stabilized by  $\text{Ca}^{2+}$  is rather stable and does not decompose even if B is removed from the medium (Fig. 3.6). This suggests that the existing pectic network does not decompose as quickly as the development of disorders. Therefore I assume that the first event following B depletion is a failure of organization of a newly secreted pectic polysaccharide as a network. Some system which is unknown at present perceive this event, and then the signal may trigger off various successive disorders. Now I am searching for the gene whose expression is induced or suppressed by the B deprivation. Identification of such genes will give a valuable information to the link between the integrity of pectic network and the cell viability.



## Conclusion

Although boron (B) is an essential microelement for higher plants, its primary function is still unknown. In this study I have isolated and characterized a B-polysaccharide complex and discussed its physiological function.

In cultured tobacco cells, at least 90% of cellular B was recovered in the cell wall preparation, while the membrane fraction did not contain a significant amount of B. When the cell wall was digested with a pectinase enzyme, about 80% of the cell wall B was recovered as the complex with a particular pectic polysaccharide. The B-polysaccharide complex (BPC) was further characterized as a candidate for the molecule in which B fulfills its physiological function.

The carbohydrate moiety of the BPC was proven to be rhamnogalacturonan II (RG-II) from its glycosyl residue- and glycosyl linkage-composition. The BPC was a dimer of RG-II cross-linked by 2 tetravalent borate esters. The B-RG-II complex could be reconstituted *in vitro* simply by mixing boric acid and RG-II at pH 4. Divalent cations such as  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Pb}^{2+}$  and  $\text{Ca}^{2+}$  significantly promoted the reconstitution by stabilizing the dimeric structure. Although  $\text{Sr}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Pb}^{2+}$  were superior to  $\text{Ca}^{2+}$  in promoting the reconstitution, they were contained in radish B-RG-II complex at less than  $0.15 \text{ mol mol}^{-1} \text{ B}$ , while  $\text{Ca}^{2+}$  was contained at  $1.2 \text{ mol mol}^{-1} \text{ B}$ . It should be  $\text{Ca}^{2+}$  that stabilizes B-RG-II *in vivo*.

As RG-II is considered to occur as a region of pectic polysaccharide in cell wall, B-RG-II complex could serve to cross-link pectic polysaccharides to form a supramolecular network. Sequential extraction showed that 80% of the pectic polysaccharides remained in cell wall even after 98% of cell wall  $\text{Ca}^{2+}$  was removed, while subsequent breakage of the B-RG-II bondings solubilized 49% of pectic polysaccharides. This suggests that not only  $\text{Ca}^{2+}$  in polygalacturonic acid region, but also B-RG-II complex contributes significantly to the cross-linking of pectic polysaccharides in cell wall. I have concluded that the physiological function of B is to build a cell wall structure by cross-linking pectic polysaccharides.





# Acknowledgements

The present thesis is based on the studies carried out at Plant Nutrition Laboratory, Graduate School of Agriculture, Kyoto University.

I wish to express my sincere gratitude to Professor Jiro Sekiya for his guidance and encouragement throughout this study.

I am deeply grateful to Dr. Toru Matoh, Associate Professor of Plant Nutrition Laboratory, for his continuous guidance and advice which enabled me to carry out this study.

I am grateful to Professor Jun-ichi Azuma, Graduate School of Agriculture, Kyoto University, for his guidance and help on instrumental analyses.

This study owes much to the preceding works on boron carried out in Plant Nutrition Laboratory. I sincerely admire the works by Dr. Nobutoshi Matsushita, Dr. Shinji Murata, Ms. Waki Matsunaga, Dr. Masaharu Mizutani, Ms. Kaori Ohno, Mr. Ken-ichi Ishigaki, Mr. Tadashi Maniwa, and Ms. Naoko Matsui. I am also indebted to my colleagues Ms. Sayaka Kawaguchi, Mr. Ryo Akaike, Ms. Miki Takasaki, Mr. Tomoyuki Asaka and Mr. Hironobu Nakagawa. Thanks also go to all the members of laboratory, for their valuable discussions.

Finally I wish to express my heartfelt thanks to my parents and my wife, for their continuous encouragement and support throughout this study.

Masaru Kobayashi



# References

- Ahmed, A. E. R. and Labavitch, J. M., 1977, A simplified method for accurate determination of cell wall uronide content. *J Food Biochem*, **1**, 361–365.
- Bjorkman, T. and Cleland, R. E., 1991, The role of extracellular free-calcium gradients in gravitropic signaling in maize roots. *Planta*, **185**, 379–384.
- Blumenkrantz, N. and Asboe-Hansen, G., 1973, New method for quantitative determination of uronic acids. *Anal. Biochem.*, **54**, 484–489.
- Bowen, H. J. M., 1966, *Trace Elements in Biochemistry*, 61–84. Academic Press.
- Brown, P. H. and Shelp, B. J., 1997, Boron mobility in plants. *Plant Soil*, **193**, 85–101.
- Cakmak, I., Kurz, H. and Marschner, H., 1995, Short-term effects of boron, germanium and high light intensity on membrane permeability in boron deficient leaves of sunflower. *Physiol. Plant.*, **95**, 11–18.
- Carpita, N. C. and Gibeaut, D. M., 1993, Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.*, **3**, 1–30.
- Da Silva, P. G. P., 1962, On the possibility of substitution of strontium for calcium in maize plants. *Agronomia Lusitana*, **24**, 133–164.
- Demarty, M., Morvan, C. and Thellier, M., 1984, Calcium and the cell wall. *Plant Cell Environ.*, **7**, 441–448.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., 1956, Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**, 350–356.
- Fischer, G. and Hecht-Buchholz, C., 1985, The influence of boron deficiency on glandular scale development and structure in *Mentha piperita*. *Planta Medica*, **5**, 371–377.
- Fry, S. C., 1986, Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Ann. Rev. Plant Physiol.*, **37**, 165–186.
- Goldbach, H. and Amberger, A., 1986, Influence of boron nutrition on cell wall polysaccharides in cell cultures of *Daucus carota* L. *J. Plant Physiol.*, **123**, 263–269.
- Gupta, U. C., 1993, Factors affecting boron uptake by plants. In U. C. Gupta, ed., *Boron and its Role in Crop Production*, chapter 5, 87–104. CRC Press, Boca Raton, Florida, USA.

- Harris, P. J., Henry, R. J., Blakeney, A. B. and Stone, B. A., 1984, An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr. Res.*, **127**, 59–73.
- Henderson, W. G., How, M. J., Kennedy, G. R. and Mooney, E. F., 1973, The interconversion of aqueous boron species and the interaction of borate with diols: A  $^{11}\text{B}$  N.M.R. study. *Carbohydr. Res.*, **28**, 1–12.
- Hirsch, A. M. and Torrey, J. G., 1980, Ultrastructural changes in sunflower root cells in relation to boron deficiency and added auxin. *Can. J. Bot.*, **58**, 856–866.
- Honda, S., Matsuda, Y., Takahashi, M., Kakehi, K. and Ganno, S., 1980, Fluorimetric determination of reducing carbohydrates with 2-cyanoacetamide and application to automated analysis of carbohydrates as borate complexes. *Anal. Chem.*, **52**, 1079–1082.
- Honda, S., Nishimura, Y., Takahashi, M., Chiba, H. and Kakehi, K., 1982, A manual method for the spectrophotometric determination of reducing carbohydrates with 2-cyanoacetamide. *Anal. Biochem.*, **119**, 194–199.
- Hu, H. and Brown, P. H., 1993, Absorption of boron by plant roots. *Plant Soil*, **193**, 49–58.
- Hu, H. and Brown, P. H., 1994, Localization of boron in cell walls of squash and tobacco and its association with pectin. Evidence for a structural role of boron in the cell wall. *Plant Physiol.*, **105**, 681–689.
- Ishigaki, K., Matoh, T., Sekiya, J. and Azuma, J., 1993, Isolation and characterization of a boron-polysaccharide complex from radish roots. *Plant Cell Physiol.*, **34**, s402.
- Ishii, T. and Matsunaga, T., 1996, Isolation and characterization of a boron-rhamnogalacturonan-II complex from cell walls of sugar beet pulp. *Carbohydr. Res.*, **284**, 1–9.
- Jarvis, M. C., 1982, The proportion of calcium-bound pectin in plant cell walls. *Planta*, **154**, 344–346.
- Jarvis, M. C., 1984, Structure and properties of pectin gels in plant cell walls. *Plant Cell Environ.*, **7**, 153–164.
- Kaneko, S., Ishii, T. and Matsunaga, T., 1997, A boron-rhamnogalacturonan-II complex from bamboo shoot cell walls. *Phytochemistry*, **44**, 243–248.
- Kouchi, H. and Kumazawa, K., 1975, Anatomical responses of root tips to boron deficiency. II. Effect of boron deficiency on cellular growth and development in root tips. *Soil Sci. Plant Nutr.*, **21**, 137–150.
- Kouchi, H. and Kumazawa, K., 1976, Anatomical responses of root tips to boron deficiency. III. Effect of boron deficiency on sub-cellular structure of root tips, particularly on morphology of cell wall and its related organelles. *Soil Sci. Plant Nutr.*, **22**, 53–71.
- Lewis, D. H., 1980, Boron, lignification and the origin of vascular plants. A unified hypothesis. *New Phytol.*, **84**, 209–229.

- Loomis, W. D. and Durst, R. W., 1992, Chemistry and biology of boron. *BioFactors*, **3**, 229–239.
- Marschner, H., 1995, *Mineral Nutrition of Higher Plants*, 2nd edition, 392–393. Academic Press, London, UK.
- Marsh, R. P. and Shive, J. W., 1941, Boron as a factor in the calcium metabolism of the corn plant. *Soil Science*, **51**, 141–151.
- Marsh, R. P., 1942, Comparative study of the calcium-boron metabolism of representative dicots and monocots. *Soil Science*, **53**, 75–78.
- Matoh, T., Akaike, R. and Kobayashi, M., 1997, A sensitive and convenient assay for boron in plant using chromotropic acid and HPLC. *Plant Soil*, **192**, 115–118.
- Matoh, T., Ishigaki, K., Mizutani, M., Matsunaga, W. and Takabe, K., 1992, Boron nutrition of cultured tobacco BY-2 cells. I. Requirement for and intracellular localization of boron and selection of cells that tolerate low levels of boron. *Plant Cell Physiol.*, **33**, 1135–1141.
- Matoh, T., Ishigaki, K., Ohno, K. and Azuma, J., 1993a, Isolation and characterization of a boron-polysaccharide complex from radish roots. *Plant Cell Physiol.*, **34**, 639–642.
- Matoh, T., Kawaguchi, S. and Kobayashi, M., 1996, Ubiquity of a borate-rhamnogalacturonan II complex in the cell walls of higher plants. *Plant Cell Physiol.*, **37**, 636–640.
- Matoh, T., Matsunaga, W., Mizutani, M. and Ohno, K., 1993b, A sensitive and microscale method for boron determination of plant materials. *Jpn. J. Soil Sci. Plant Nutr.*, **64**, 71–73.
- Matoh, T., Takasaki, M., Kawaguchi, S. and Kobayashi, M., 1998, Immunocytochemistry of rhamnogalacturonan II in cell walls of higher plant. *Plant Cell Physiol.*, **39**, 483–491.
- Matoh, T., Takasaki, M., Kobayashi, M. and Takabe, K., 2000, Boron nutrition of cultured tobacco BY-2 cells. III. Characterization of the boron-rhamnogalacturonan II complex in cells acclimated to low levels of boron. *Plant Cell Physiol.*, **41**, 363–366.
- Matoh, T., 1997, Boron in plant cell walls. *Plant and Soil*, **193**, 59–70.
- Matsunaga, T., Ishii, T. and Watanabe-Oda, H., 1997, HPLC/ICP-MS study of metals bound to borate-rhamnogalacturonan-II from plant cell walls. In T. Ando, K. Fujita, T. Mae, H. Matsumoto, S. Mori and J. Sekiya, eds., *Plant Nutrition: for Sustainable Food Production and Environment*, 81–82. Kluwer Academic Publishers, Netherlands.
- Melton, L. D., McNeil, M., Darvill, A. G., Albersheim, P. and Dell, A., 1986, Structural characterization of oligosaccharides isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydr. Res.*, **146**, 279–305.
- Mizutani, M., 1992, Studies on physiological function of boron in higher plants. Master's thesis, Graduate School of Agriculture, Kyoto University.

- Mühling, K. H., Wimmer, M. and Goldbach, H. E., 1998, Apoplastic and membrane-associated  $\text{Ca}^{2+}$  in leaves and roots as affected by boron deficiency. *Physiol. Plant.*, **102**, 179–184.
- Nagata, T., Okada, K., Takebe, I. and Matsui, C., 1981, Delivery of tobacco mosaic virus RNA into plant protoplasts mediated by reverse-phase evaporation vesicles (liposomes). *Mol. Gen. Genet.*, **184**, 161–165.
- Oi, T., Tanaka, T. and Kakihara, H., 1992, Esterification of boric acid with 1,2-propanediol, 3-amino-1,2-propanediol, and ( $\pm$ )-3-dimethylamino-1,2-propanediol as studied by  $^{11}\text{B}$  NMR spectroscopy. *Bull. Chem. Soc. Jpn.*, **65**, 1903–1909.
- O'Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G. and Albersheim, P., 1996, Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plants cells, forms a dimer that is covalently cross-linked by a borate ester. In vitro conditions for the formation and hydrolysis of the dimer. *J. Biol. Chem.*, **271**, 22923–22930.
- Parr, A. J. and Loughman, B. C., 1983, Boron and membrane function in plants. In Robb, D. A. and Pierpoint, W. S., editors, *Metals and Micronutrients: Uptake and Utilization by Plants*, 87–107. Academic Press, New York.
- Pellerin, P., Doco, T., Vidal, S., Williams, P., Brillouet, J.-M. and O'Neill, M., 1996, Structural characterization of red wine rhamnogalacturonan II. *Carbohydr. Res.*, **290**, 183–197.
- Pollard, A. S., Parr, A. J. and Loughman, B. C., 1977, Boron in relation to membrane function in higher plants. *J. Exp. Bot.*, **28**, 831–841.
- Puvanesarajah, V., Darvill, A. G. and Albersheim, P., 1991, Structural characterization of two oligosaccharide fragments formed by the selective cleavage of rhamnogalacturonan II: evidence for the anomeric configuration and attachment sites of apiose and 3-deoxy-2-heptulosaric acid. *Carbohydr. Res.*, **218**, 211–222.
- Schmucker, T., 1933, Zur Blütenbiologie Tropischer Nymphaea-Arten. II. (Bor als Entscheidender Faktor). *Planta*, **18**, 641–650.
- Skok, J. and McIlrath, W. J., 1958, Distribution of boron in cells of dicotyledonous plants in relation to growth. *Plant Physiol.*, **33**, 428–431.
- Skok, J., 1957, The substitution of complexing substances for boron in plant growth. *Plant Physiol.*, **32**, 308–312.
- Slack, C. R. and Whittington, W. J., 1964, The role of boron in plant growth. III. The effects of differentiation and deficiency on radicle metabolism. *J. Exp. Bot.*, **15**, 495–514.
- Smith, M. E., 1944, The role of boron in plant metabolism. 1. Boron in relation to the absorption and solubility of calcium. *Aust. J. Exp. Biol. Med. Sci.*, **22**, 257–263.
- Sommer, A. L. and Lipman, C. B., 1926, Evidence on the indispensable nature of zinc and boron for higher green plants. *Plant Physiol.*, **1**, 231–249.

- Spellman, M. W., McNeil, M., Darvill, A. G. and Albersheim, P., 1983, Characterization of a structurally complex heptasaccharide isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydr. Res.*, **122**, 131–153.
- Spurr, A. R., 1957, The effect of boron on cell-wall structure in celery. *Am. J. Bot.*, **44**, 637–650.
- Stevenson, T. T., Darvill, A. G. and Albersheim, P., 1988, Structural features of the plant cell wall polysaccharide rhamnogalacturonan-II. *Carbohydr. Res.*, **182**, 207–226.
- Sweet, D. P., Shapiro, R. H. and Albersheim, P., 1975, Quantitative analysis by various g.l.c. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydr. Res.*, **40**, 217–225.
- Talbott, L. and Ray, P., 1992, Molecular size and separability features of pea cell wall polysaccharides. *Plant Physiol.*, **98**, 357–368.
- Teasdale, R. D. and Richards, D. K., 1990, Boron deficiency in cultured pine cells: Quantitative studies of the interaction with Ca and Mg. *Plant Physiol.*, **93**, 1071–1077.
- Thomas, J. R., Darvill, A. G. and Albersheim, P., 1989, Isolation and structural characterization of the pectic polysaccharide rhamnogalacturonan II from walls of suspension-cultured rice cells. *Carbohydr. Res.*, **185**, 261–277.
- Torssell, K., 1956, Chemistry of arylboric acids. VI. Effects of arylboric acids on wheat roots and the role of boron in plants. *Physiol. Plant.*, **9**, 652–664.
- Van Duin, M., Peters, J. A., Kieboom, A. P. G. and Van Bakkum, H., 1987, Synergic coordination of calcium in borate-polyhydroxycarboxylate systems. *Carbohydr. Res.*, **162**, 65–78.
- Warington, K., 1923, The effect of boric acid and borax on the broad bean and certain other plants. *Ann. Bot.*, **37**, 629–672.
- Yamanouchi, M., 1971, The role of boron in higher plants I. The relations between boron and calcium or the pectic substance in plants. *J. Soil Sci. Manure Jpn.*, **42**, 207–213.
- Yamauchi, T., Hara, T. and Sonoda, Y., 1986, Distribution of calcium and boron in the pectin fraction of tomato leaf cell wall. *Plant Cell Physiol.*, **27**, 729–732.
- York, W. S., Darvill, A. G., McNeil, M. and Albersheim, P., 1985, 3-Deoxy-D-manno-2-octulosonic acid (KDO) is a component of rhamnogalacturonan II, a pectic polysaccharide in the primary cell walls of plants. *Carbohydr. Res.*, **138**, 109–126.
- York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T. and Albersheim, P., 1986, Isolation and characterization of plant cell walls and cell wall components. *Method. Enzymol.*, **118**, 3–41.





# Publications

- Kobayashi, M., Matoh, T., and Azuma, J. (1996) Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiol.*, **110**, 1017-1020.
- Kobayashi, M., Ohno, K., and Matoh, T. (1997) Boron nutrition of cultured tobacco BY-2 cells. II. Characterization of the boron-polysaccharide complex. *Plant Cell Physiol.*, **38**, 676-683.
- Kobayashi, M., and Matoh, T. (1997) *In vitro* reconstitution of the boron-polysaccharide complex purified from cultured tobacco BY-2 cells. In R. W. Bell and B. Rerkasem eds., *Boron in Soils and Plants*, 237-241. Kluwer Academic Publishers, Netherland.
- Matoh, T., and Kobayashi, M. (1998) Boron and calcium, essential inorganic constituents of pectic polysaccharides in higher plant cell walls. *J. Plant Res.*, **111**, 179-190.
- Kobayashi, M., Nakagawa, H., Asaka, T., and Matoh, T. (1999) Borate-rhamnogalacturonan II bonding reinforced by  $\text{Ca}^{2+}$  retains pectic polysaccharides in higher-plant cell walls. *Plant Physiol.*, **119**, 199-203.